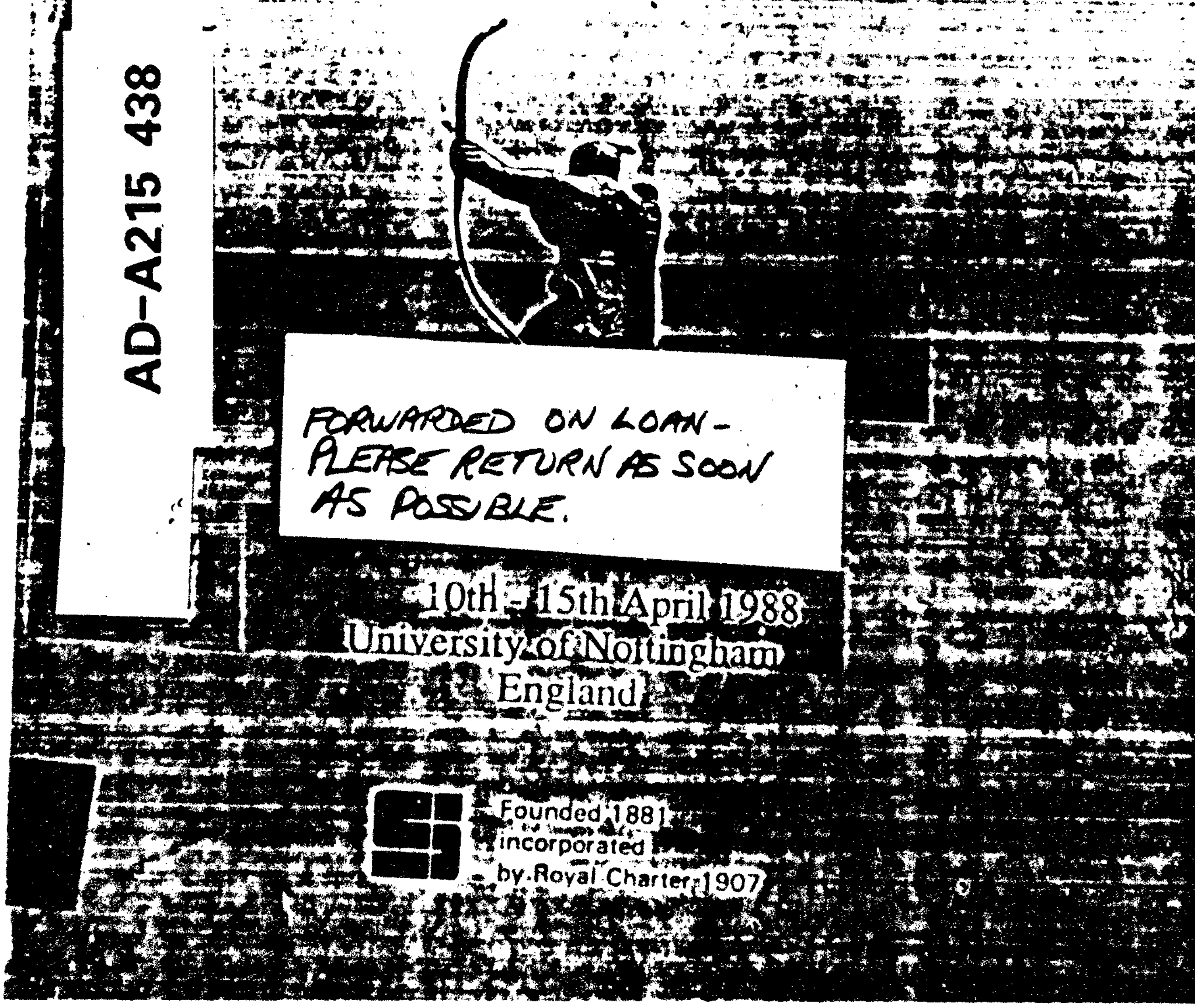
20030204026

The Society of Chemical Industry

NEUROTOX '88

Molecular Basis of Drug & Pesticide Action

ABSTRACTS



AD-A215 438

FORWARDED ON LOAN -
PLEASE RETURN AS SOON
AS POSSIBLE.

10th - 15th April 1988
University of Nottingham
England

Founded 1881
incorporated
by Royal Charter 1907

PHOTOGRAPH THIS SHEET

AD-A215 438

DTIC ACCESSION NUMBER

LEVEL

R/D 5986-BC-02

INVENTORY 1

DTIC FILE COPY

Molecular Basis of Drug & Pesticide Action Abstracts April 1988

DOCUMENT IDENTIFICATION

DAJA45-88-M-0086

DISTRIBUTION STATEMENT A

Approved for public release;
Distribution Unlimited

DISTRIBUTION STATEMENT

ESSION FOR
GRA&I ☒
TAB ☐
NNOUNCED ☐
TIFICATION per form

RIBUTION /
ILABILITY CODES
AVAIL AND/OR SPECIAL

-1

DISTRIBUTION STAMP

DTIC
ELECTE
DEC 14 1989
S B D

DATE ACCESSIONED

DATE RETURNED

89 12 12 011

DATE RECEIVED IN DTIC

REGISTERED OR CERTIFIED NO.

PHOTOGRAPH THIS SHEET AND RETURN TO DTIC-FDAC

ORM 70A
AR 86

DOCUMENT PROCESSING SHEET

PREVIOUS EDITION MAY BE USED UNTIL

The organisers would like to thank
the following organisations for
generous donations received to date:

American Cyanamid Co (USA)

A/S Cheminova (Denmark)

Bayer Service Co (Egypt)

British Crop Protection Council (UK)

Ciba-Geigy Ag (Switzerland)

Dow Chemical (USA)

E I du Pont de Nemours (USA)

ICI/Plant Protection (Egypt)

Institut Scientifique Roussel (France)

Meiji Seika Kaisha Ltd (Japan)

Merck & Co Inc (USA)

Mitsubishi Chemical Industries Ltd (Japan)

Nippon Chemiphar Co Ltd (Japan)

Rhone-Poulenc AG Co (USA)

Rohm and Haas Co (USA)

US Department of Army

Sandoz Ltd (Switzerland)

Schering Agrochemicals Ltd (UK)

Shell Research Ltd (UK)

Takeda Chemical Industries Ltd (Japan)

Teijin Ltd (Japan)

University of Maryland (USA)

Wellcome Research Laboratories (UK)

ABSTRACTS OF PAPERS PRESENTED AT NEUROTOX '88

APRIL 10-15, 1988

INTRODUCTION

The abstracts presented in this book are organised in two Sections:

Section I contains abstracts of talks presented by invited speakers. These abstracts are arranged in the order in which the talks will be presented.

Section II contains abstracts related to poster presentations. Posters have been assigned to one of four sections:

A - NATURAL PRODUCTS AND NEUROPEPTIDES

B - PHYSIOLOGICAL ASPECTS OF RECEPTORS AND CHANNELS

C - BIOCHEMISTRY AND MOLECULAR BIOLOGY OF NEUROTRANSMISSION

D - DRUGS AND PESTICIDES: MOLECULAR BASIS OF SELECTIVITY, ACTION AND RESISTANCE.

Within these sections abstracts are arranged alphabetically by first author. Numbers A1 etc. refer to poster locations at the poster exhibition.

There are a number of blank pages included for your personal use.

Abstracts presented in this book include all submissions which reached the editor at 1st March 1988.

Finally the organisers would like to thank authors for the careful presentation and prompt submission of their abstracts. We hope you will find this book useful.

SECTION I - CONTENTS

A.M. Monday - 11th April

6 Michael Adams Spider Toxins affecting multiple targets at the insect neuromuscular junction.

7-8 Tom Piek, Henk Karst, Cor Kurk, Albert Lind, Jan van Marle, Terumi Nakajimi, Nico Nibbering, Haruhiko Shinozaki, Willem Spanjer and Yulan Tong. Polyamine-like toxins - A new class of pesticides?

P.M. Monday - 11th April

PLENARY LECTURE

9-10 Terumi Nakajima, Tadashi Yasuhara and Nobofumi Kawai. Animal toxins of low molecular mass.

NATURAL PRODUCTS - PART II

11 Michael O'Shea, Siegfried Hekimi and Marie-Francoise Schulz. Insect peptide hormone biosynthesis.

12-13 Ian Duce. Spider venoms as probes for insect neurotransmission.

14 Hunter Jackson and Thomas Parks. Effects of spider venoms on the vertebrate nervous system.

15 E. K. Michaelis. Purification of an inhibitor of brain synaptic membrane glutamate binding sites from the venom of the spider *Araneus gemma*.

16-17 Haruhiko Shinozaki. Novel excitatory amino acid related compounds of natural origin.

18 Haruhisa Shirahama. Acromelic acid A and B, very potent excitatory amino acids from a poisonous mushroom.

A.M. Tuesday - 12th April

PLENARY LECTURE

19 John Casida, Russell Nicholson and Christopher Palmer. Trioxabicyclooctanes as probes for the convulsant site of the GABA-gated chloride channel.

GABA RECEPTORS

20-21 P. Krogsgaard-Larsen, L. Nielsen, J.S. Jensen, E. Falch, L. Brehm and F.S. Jorgensen. Molecular pharmacology of GABA synaptic mechanisms.

22 Y. Aracava, R. Rosental, K.L. Swanson, H. Rapoport and E.X. Alberquerque. Structure-activity relationships of (+) anatoxin -a derivatives and enantiomers of nicotine on the peripheral and central nicotinic acetylcholine receptor subtypes.

23 George Lunt. The biochemical characterisation of insect GABA receptors.

24 Jack Benson. Transmitter receptors on insect neuronal somata: GABA-ergic and cholinergic pharmacology.

25 Mohyee Eldefrawi and Amira Eldefrawi. Action of toxicants on GABA and glutamate receptors.

P.M. Tuesday - 12th April

PLENARY LECTURE

R.H. Michell. Receptor mediated signalling using second messengers.

SECOND MESSENGERS

26-27 Peter Evans. Second messenger systems in insects: an introduction.

28 Fumio Matsumura. Effects of pyrethroids on neural protein kinases and phosphatases.

29-30 Michael Duggan and George Lunt. Coupling of muscarinic receptors to second messenger systems in locust ganglia.

31-32 James Nathanson. Octopamine receptors: Biochemistry and mode of action.

33-34 Roger Downer. Octopamine and dopamine-sensitive receptors and cyclic AMP production in insects.

A.M. Wednesday - 13th April

PLENARY LECTURE

35-36 Toshio Narahashi. Molecular and cellular approaches to neurotoxicology: past, present and future.

MOLECULAR NEUROBIOLOGY

37-38 Oliver Dolly. Functional components in the nervous system revealed by inhibitory and facilitatory toxins.

39-40 Heinz Breer. Receptors for acetylcholine in the nervous system of insects.

41 C.F. Wu. Genetic and pharmacological analyses of potassium channels in *Drosophila*.

42-43 Y. Pichon. Acetylcholine, GABA and glutamate receptor channels in cultured insect neurones.

44 Paul Salvaterra. Molecular Biology of *Drosophila* choline acetyltransferase.

A.M. Thursday - 14th April

PLENARY LECTURE

Edson X. Alberquerque. The role of carbamates and oximes in reversing toxicity of organophosphorus compounds. A perspective into mechanisms.

RECEPTORS AND ION CHANNELS

45-46 Daisuke Yamamoto. Patch-clamp analysis of single chloride channels in primary neuronal cultures of *Drosophila*.

47-48 Cathryn J. Kerry and Peter N.R. Usherwood. Single channel studies of the interactions of $Agriotoxin_{636}$ with locust muscle glutamate receptors.

49-50 Alistair Mathie. Patch-clamp recordings of transmitter-activated ion channels in mammalian sympathetic neurones.

51-52 J. Dudel. Glutamate activated membrane channels in crustaceans.

52-53 Mark Sansom. Ion channels in artificial lipid bilayers.

P.M. Thursday - 14th April

PLENARY LECTURE

54 P.M. Dean. Molecular recognition, structural dissimilarity and future developments in quantitative structure activity relationships - (QSAR).

STRUCTURE-ACTIVITY RELATIONSHIPS

55-56 Jeffrey Watkins. Structure-activity studies on mammalian glutamate. receptors.

57-58 David Salt and Martyn Ford. . The use of multivariate analysis in toxicological studies.

59-60 David Livingstone, Martyn Ford and David Buckley. A multivariate QSAR study of pyrethroid neurotoxicity based upon molecular parameters derived by computational chemistry.

61-62 Fleming Jorgensen, Jette Byberg, Povl Krogsgaard-Larsen and James Snyder. Qualitative and quantitative modelling of drugs and pesticides.

A.M. Friday - 15th April

PLENARY LECTURE

R.M. Hollingworth

SELECTIVITY AND RESISTANCE

63-64 Martyn Ford. The dynamic basis of selective toxicity.

SECTION II - ABSTRACTS OF CONTRIBUTED PAPERS

A - NATURAL PRODUCTS AND NEUROPEPTIDES

89-90 A10 - Henk KARST, Albert LIND, Jan van MARLE, Terumi NAKAJIMA, Tom PIEK and Janneke van WEEREN-KRAMER. Effects of spider toxins on glutamate transmission and uptake.

91 A11 - B.G. LOUGHTON. The mode of action of locust hypolipaemic hormone.

92 A12 - Prem N. MISHRA. Studies on ovicidal properties of some natural products on the eggs of *Henosepilachra vigintioctopunctata*.

93 A13 - MORDUE (LUNTZ), A.J. and PLANE, F. Muscular effects of azadirachtin.

94-95 A14 - Terumi NAKAJIMA, Tom PIEK, Tadashi YASUHARA and Piet MANTEL. Kinins isolated from the venom of the solitary wasp *Megascolia flavifrons*.

96-97 A15 - Russell A. NICHOLSON, Philip S. ROBINSON, Christopher J. PALMER and John E. CASIDA. Ivermectin-stimulated release of neurotoransmitter in the insect central nervous system: Modulation by external chloride and inhibition by a novel trioxabicyclooctane and two polychlorocycloalkane insecticides.

98-99 A16 - Keiichiro NISHIMURA, Takamitsu KOBAYASHI and Toshio FUJITA. Physicochemical properties of pyrethroids and sodium currents in crayfish giant axon.

100-101 A17 - David R. PEPPER and Ian R. DUCE. A comparison of the effects of venoms from four spider families on neuromuscular synapses of insects.

102 A18 - Tom PIEK, Bernard HUE, Albert LIND, Piet MANTEL, Jan van MARLE and Hans H. VISSEP. *Ampulex compressa* changes the behaviour of the cockroach.

103 A19 - Tom PIEK, Bernard HUE, Piet MANTEL, Terumi NAKAJIMA, Marcel PELHATE and Tadashi YASUHARA. Effects of the venom of *Copa interrupta* on vertebrate smooth muscle and synaptic transmission in insect CNS.

104 A20 - Stuart E. REYNOLDS, Richard I. SAMUELS and A. Keith CHARNLEY. Destruxins, fungal toxins that activate calcium channels in insect muscle.

105-106 A21 - Sandra ROBB and Peter D. EVANS. Distribution of FMRFamide-life peptides in the locust nervous system.

107-108 A22 - James M. SCHAEFFER, Herbert W. HAINES and Mervyn J. TURNER. Avermectin binding in *Caenorhabditis elegans*: A two-stated model for the avermectin binding site.

109-110 A23 - James M. SCHAEFFER, Thomas L. SHIH, Mervyn J. TURNER and Helmut MROZIK. Agriopine differentiates between vertebrate and invertebrate glutamate binding sites.

B - PHYSIOLOGICAL ASPECTS OF RECEPTORS AND ION CHANNELS

C - BIOCHEMISTRY AND MOLECULAR BIOLOGY OF NEUROTRANSMISSION

APPENDIX

241	Michel LAZDUNSKI. Molecular biology and pharmacology of ionic channels.
242-243	AX.– G.S. BESWICK and G.A. COTTRELL. Differential effects of FRMFamide peptides in *Helix* tentacle muscle.

SECTION I

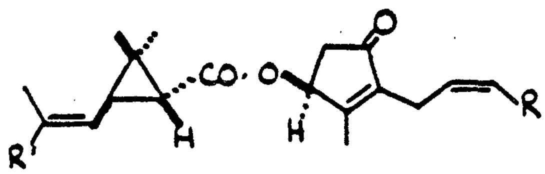
****

ABSTRACTS OF INVITED CONTRIBUTIONS

THE NATURAL PYRETHRINS : A CHEMIST'S VIEW

Leslie Crombie
Department of Chemistry, The University of Nottingham, Nottingham, NG7 2RD.

The development of the structure, stereochemistry and synthesis of the group of natural insecticides from the achenes of the flower heads of Chrysanthemum cinerariaefolium (pyrethrum), collectively known as the pyrethrins, will be discussed. The



Pyrethrin	I	R = $CH{=}CH_2$	R'=Me
"	II	R = $CH{=}CH_2$	R'=CO_2Me
Cinerin	I	R = Me	R'=Me
"	II	R = Me	R'=CO_2Me
Jasmolin	I	R = CH_2Me	R'=Me
"	II	R = CH_2Me	R'=CO_2Me

six esters are shown above and have become the natural models from which todays' commercial pyrethroid insecticides [e.g. allethrin, (S)-bioallethrin, resmethrin, bioresmethrin, permethrin, cypermethrin, deltamethrin, fenvalerate etc.] have developed, though the natural pyrethrins themselves continue to have a place in the market.

The biosynthetic origins of the pyrethrins is still poorly documented although structures similar to chrysanthemic acid are found in the important compounds presqualene and prephytoene. Rethrolone esters of linoleic and palmitic acids occur in pyrethrum extract and the origin of the rethrolones may well lie in the 12-oxophytodienoic acid (allene epoxide) pathway now emerging in higher plants.

EFFECTS OF KININS AND RELATED PEPTIDES ON SYNAPTIC TRANSMISSION IN THE INSECT CNS.

Bernard Hue* and Tom Piek+

* Department of Neurophysiology, UA CNRS n° 611, The University of Angers, 49045 Angers Cedex, France.

+ Department of Pharmacology, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands.

In three taxonomically related groups of Hymenoptera, i.e., the social wasps, the scoliid wasps and the ants, a characteristic type of polypeptide toxins have been found,[1,2,3] the action of which on vertebrate smooth muscle, as well as the chemical structure are closely related to that of bradykinin. The bradykinin-like peptides have been called kinins by Jacques and Schachter[4]. Because scoliid wasps use their venom to paralyse beetle larvae by stinging them in the nerve ganglia[5], we have studied the effects of the venom of the wasp Megascolia flavifrons and its fractions is comparison with those of bradykinin and Thr[6]bradykinin on cholinergic synaptic transmission between cercal afferent fibres and giant interneurons in the sixth abdominal ganglion of the cockroach Periplaneta americana.

Electrophysiological recordings were obtained, using the single-fibre oil-gap method[6]. A mannitol-gap method was also adapted to the presynaptic cercal afferents, thus allowing the presynaptic polarization to be appreciated together with the postsynaptic one. The venom and toxins were applied by superfusion or by perfusion, using pressure application through a micropipette inserted within the neuropile of the desheathed sixth abdominal ganglion. The effects were appreciated as modifications of synaptic efficiency, i.e., : EPSP amplitude, ionophoretically-induced carbamylcholine potentials, pre- and postsynaptic resting potential, membrane conductance and excitability.

The venom and the Thr^6bradykinin containing fraction, as well as bradykinin (10^{-4} M) cause a very progressive block of synaptic transmission without any change in postsynaptic membrane conductance and axonal excitability.

This blocking action was localized at the presynaptic level as it was suggested by the following experimental data.

During perfusion with Thr^6bradykinin (5×10^{-5} M) the EPSP amplitude decreased gradually. However, the carbamylcholine-induced potential was not affected. Moreover, t' stimulus-dependent decrease in EPSP-amplitude during a pulse train of low frequency strongly suggests a depletion of readily releasable transmitter during low frequency release. The fact, that the readily releasable store is partly replenished between pulse trains, indicates that kinins may affect the transport system rather than the storage of transmitter. It may be evident, from this study, that kinins or kinin-like peptides play an important role in the long term paralysis caused by stings of the wasp into the insect CNS.

References

1. Piek, T. In "Venom of the Hymenoptera, Biochemical, Pharmacological and Behavioural Aspects", Academic Press, London, 1986, 1-570.
2. Yasuhara, T., Mantel, P., Nakajima, T. and Piek, T. Toxicon, 1987, 25, 527-535.
3. Piek, T., Schmidt, J.O., de Jong, J.M. and Mantel, P. Comp. Biochem. Physiol. 1988 (in preparation).
4. Jacques, R. and Schachter, M. Br. J. Pharmacol., 1954, 9, 53-57.
5. Piek, T., Buitenhuis, A., Simonthomas, R.T., Ufkes, J.G.R. and Mantel, P. Comp. Biochem. Physiol., 1983, 75C, 145-152.
6. Callec, J.J., Sattelle, D.B., Hue, B. and Pelhate M. In "Insect Neurobiology and Pesticide Action", Soc. Chem. Industry ed. London, 1980, 93-100.

ON THE SITE OF ACTION OF THE INSECT SELECTIVE NEUROTOXIN - AaIT.

Eliahu Zlotkin[1], Lena Fishman[1] and Dalia Gordon[2].
[1] Life Sciences, Zoology, The Hebrew University, Jerusalem 91904, Israel, [2] Department of Pharmacology, SJ-30, University of Washington, Seattle, WA 98195, USA.

The AaIT is a neurotoxic polypeptide (M_r 8 kD) derived from scorpion venom which selectively affects insects by the induction of a fast excitatory paralysis[1]. The present study deals with (a) the penetrability of the AaIT and (b) the essence of its receptor.

(a) The first aspect is dedicated to the question how does a relatively polar and large molecule such as the AaIT reach the well protected membranes of the insect nervous tissue? This aspect was treated by LM autoradiography and binding assays resulting in the following information: (1) Dissected insect nerves (with affected enveloping tissues) have revealed a specific binding of a high affinity of the [^{125}I]AaIT at the various regions and outbranchings of the central as well as peripheral nervous system of insects. (2) Injection of [^{125}I]AaIT into the insect body cavity (thus mimicking a natural scorpion sting), in a dose inducing motor disturbances of the animal, has revealed that the CNS and the vast majority of the peripheral branches are impermeable to the toxin. The toxin, however, was shown to bind to the terminal branches of motor nerves at their close proximity to the skeletal muscles. (3) The above data coupled with binding studies suggest that the sensitivity of the insect to the AaIT is not dependent only on its apparent binding affinity (K_D) to the isolated neuronal membranes but rather on the accessibility of the nervous tissues to the toxin.

(b) The second aspect is dedicated to the clarification of the pharmacological identity of the AaIT receptor. The following series of evidence suggests that the AaIT interacts with the insect neuronal sodium channel: (1) AaIT possesses binding constants and pharmacologic characteristics typical to the β-scorpion toxins affecting vertebrates which are well known markers of Na^+ channels[2]. (2) A β-scorpion toxin was recently shown to possess identical binding sites to those of AaIT in an insect synaptosomal preparation[3]. (3) The AaIT has revealed an identical binding capacity to that of saxitoxin[4]. (4) Voltage clamp studies with an isolated nerve fibre of an insect have

clearly indicated that AaIT has exclusively affected sodium conductance[5]. With this background the sodium channel of a locust CNS has recently[6] been identified and characterized through the employment of antibodies against a peptide (SPI9) corresponding to a highly conserved predicted intracellular region of a sodium channel α-subunit. The channel protein was visualized on an SDS-PAGE through its phosphorylation [^{32}P] of the Sepharose protein A precipitate of the channel-antibody complex. It has been shown that the α-subunit of the locust channel (1) has a molecular weight of about 280 kD; (2) serves as a substrate of phosphorylation by cAMP-dependent protein kinase; (3) is devoid of a disulphide linkage to β subunits and (4) is devoid of sialic acid. These data supply the theoretical and methodological basis for a further study of the interaction of AaIT with the insect sodium channel.

References

1. Zlotkin, E. In Pharmacology and Pesticide Action, Horwood, 1986 352-383.

2. Gordon, D., Jover, E., Couraud, F. and Zlotkin, E. BBA 1984 778 349-358.

3. De Lima, M.E., Martin, M.F., Diniz, C.R. and Rochat, H. BBRC 1986 139 296-302.

4. Gordon, D., Zlotkin, E. and Catterall, W.A. BBA 1985 821 130-136.

5. Pelhate, M. and Zlotkin, E. J. Physiol. Lond. 1981 319 30-31.

6. Gordon, D., Merrick, D., Wollner, D.A. and Catterall, W.A. Biochemistry. In press.

Spider Venom Toxins Affecting Multiple Targets at the Insect Neuromuscular Junction

Michael E. Adams

Division of Toxicology and Physiology, Department of Entomology, University of California, Riverside, CA 92521

Araneid (*Argiope*, *Araneus*) and Agelenid (*Agelenopsis*) spider venoms show clear differences in biochemistry and physiological actions. These differences relate to functionally distinct classes of venom toxins affecting neuromuscular transmission. Non-lethal, flaccid paralysis caused by Araneid venoms disappears within hours and is attributed to reversible postsynaptic toxins. *Agelenopsis* venom induces irreversible paralysis associated with an initial period of excitation. Here, postsynaptic toxins resembling those of the Araneids act in concert with excitatory and inhibitory presynaptic toxins exerting long lasting disruption of transmitter release. Presynaptic toxins in *Agelenopsis* venom, while intrinsically active alone, act to synergize the paralytic activity of the postsynaptic toxins.

All three venoms contain low molecular weight (400-800 daltons) postsynaptic toxins which block glutamate-sensitive receptor channels in muscle. Inhibition of neurally-evoked EPSP's and iontophoretic glutamate potentials is stimulus-dependent and follows two distinct time courses, a fast block and a slow block. The toxins show a common structural motif consisting of a basic, positively charged region coupled to a relatively hydrophobic aryl moiety. *Argiope* toxins ("argiotoxins") and *Araneus* toxins ("aranetoxins") incorporate arginine (free amino) and asparagine with unconventional polyamines to form the basic part of the molecule. *Agelenopsis* toxins (α-agatoxins) lack amino acids, but have extended polyamines. Aryl components in all cases are contributed by indolic or phenylacetic acids. Variation of polyamine configuration in native argiotoxins toxins correlates with potency differences in both paralysis and neuromuscular assays. Hydroxylation of the aryl moiety may influence the reversibility of synaptic block.

Agelenopsis venom contrasts with Araneid venoms in having two additional classes of toxins affecting transmitter release. We have isolated six excitatory peptides (μ-Agatoxins I-VI) which cause repetitive firing in nerve terminals reminiscent of scorpion toxins. These are 36-37 amino acids long, including eight cysteine residues, and hence are presumed to contain four disulfide bridges. The μ-Agatoxins synergize postsynaptic block caused by the α-agatoxins, apparantly by causing massive release of natural transmitter from nerve terminals. Additional toxins in *Agelenopsis* venom cause irreversible block of EPSP's without affecting iontophoretic glutamate potentials.

POLYAMINE LIKE TOXINS - A NEW CLASS OF PESTICIDES?

Tom Piek[1], Henk Karst[1], Cor Kruk[3], Albert Lind[1], Jan van Marle[2], Terumi Nakajima[4], Nico M.M. Nibbering[3], Haruhiko Shinozaki[5], Willem Spanjer[1] and Yulan C. Tong[6].

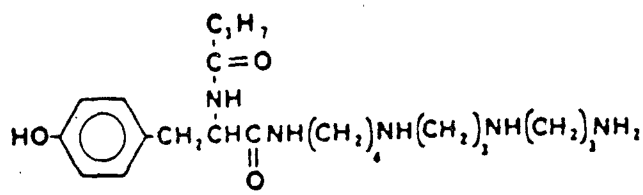
Departments of Pharmacology[1], Electronmicroscopy[2], Meibergdreef 15, 1105 AZ Amsterdam, Organic Chemistry[3], Nieuwe Achtergracht 129, 1018 WS Amsterdam, The Netherlands, Faculty of Pharmaceutical Sciences[4], and the Tokyo Metropolitan Inst. of Med.Sc.[5], Bunkyo-ku, Tokyo 113, Japan, and Dow Chemical Res.C. Walnut Creek, California U.S.A.[6]

Venoms of some species of spiders and wasps contain a number of toxins, which block glutamatergic transmission[1-8]. The toxic principles are peptid-polyamine structures in both spider venom[6-9] and wasp venom (this paper). We have compared the effects, on insect neuromuscular transmission, of the synthetic toxins NSTX-3 (from the spider Nephila maculata) and JSTX-3 (from N.clavata), δ-PTX (from the wasp Philanthus triangulum) with the synthetic polyamines MLV-5860 and MLV-6976, which also affect the glutamatergic transmission[10].

All toxins block glutamate potentials evoked by iontophoretically applied glutamate pulses. They are active at the following concentrations: 10^{-8} M (NSTX-3, JSTX-3), 10^{-7} M (δ-PTX), 10^{-5} M (MLV-5860) and 10^{-5} M (MLV-6976). In this order the speed of reversibility increases. Concanavalin A considerably increases the reversibility of NSTX-3.

The spider toxins and δ-PTX curtail glutamate potentials, the MLV's do not so.

The high affinity uptake of glutamate by terminal axons and glial cells is significantly increased by JSTX-3 and NSTX-3, significantly inhibited by δ-PTX, and not affected by the MLV's.



S-PTX
(Philanthus triangulum)

References

1. Kawai, N., Niwa, A. and Abe, T. Brain Res. 1982, 247, 169-171.
2. Abe, T., Kawai, N. and Niwa, A. J.Physiol. (Lond.) 1983, 339, 243-252.
3. Bateman, A. Boden, P., Dell, A. Duce, I.R., Quicke, D.L.J. and Usherwood, R.N.R. Brain Res.1985, 339, 237-244.
4. Clark, R.B., Donaldson, P.L., Gration, K.A.F., Lambert, J.J., Piek, T., Ramsey, R., Spanjer, W. and Usherwood, P.N.R. Brain Res. 1982, 241, 105-114.
5. Piek, T. Comp.Biochem.Physiol.1982, 72C, 311-315.
6. Volkova, T.M., Grishin, E.V., Arseniev, A.S., Reshetova, O.S., Onoprienko, V.V. Proc.6th Europ.Soc.Neurochem.Meet.1986, 189.
7. Aramaki, Y., Yasuhara, T., Higashijima, T., Yoshioka, M., Miwa, A., Kawai, N. and Nakajima, T. Proc.Japan Acad.1986, 62B, 359-362.
8. Aramaki, Y., Yasuhara, T., Shimazaki, K., Kawai N. and Nakajima, T. Biomed.Res. 1987, 8, 241-245.
9. Nakajima, T. and Kawai, N. Abstr. 1st Asia-Pacific Congr. Anim.Plant. Microb.Toxins, Singapore 1987, 0059.
10. Shinozaki, J. and Ishida, M., Brain Res.1986, 372, 260-268.

ANIMAL TOXINS OF LOW MOLECULAR MASS.

Terumi Nakajima, Tadashi Yasuhara and Nobufumi Kawai*

Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo 113, and
*Tokyo Metropolitan Institute of Neurosciences, Fuchu, Tokyo 183, Japan

We have characterized novel toxic components of low molecular mass during the elucidating process of the biologically active substance in animal origin.

Novel cardiac steroids in the snake. The snake belonging to the genus *Rhabdophis* has a pair of special glands, nuchodorsal glands, along the neck. The material in these glands was reported to cause severe injury when splashed into eyes by careless handling of the snake or by beating the snake's neck with a club. We analyzed this glandular material chemically and demonstrated the presence of novel polyhydroxylated cardiac steroids[1]. It is well known that bufonid toads contain cardiac steroids in the prominent parotoid glands located behind the eyes. Different from those in toad, cardiac steroids in the snake occur in a non-conjugated form in the glands and are characteristically tetrahydroxylated constitutents with respect to their common features, i.e. 3,5,11,14; 3,7,11,14 or 3,5,14,16 respectively. Trihydroxylated steroid, gamabufotalin is also present. Some snake cardiac steroids were as potent as ouabain in the positive inotropic action on guinea pig papillary muscle.

Novel cytotrophic peptides in wasp venom. The social wasps commonly possess two series of the hydrophobic peptide family as the venom components. One of them are histamine releasing peptides, mastoparans, and others are vespid chemotactic peptides. Both families of peptides are composed of hydrophobic amino acids and basic amino acid[2]. Among these peptides, mastoparan shows a variety of biological action in addition to mast cell degranulation and histamine release. Mastoparan acts on adrenal chromaffin cells, platelets to release catecholamines or serotonin, and raises prolactin secretion from pituitary cells. It was also reported that mastoparan bound to calmodulin in the presence of Ca ion with the highest affinity and inhibited the calmodulin-sensitive enzymes as phosphodiesterase. Mastoparan activates membrane bound phospholi-

pase A_2. In addition, mastoparan activates GTP-binding protein in a cell membrane to stimulate the binding rate of GTP to the protein. vespid chemotactic peptide is a tridecapeptide amide which causes chemotaxis for polymorphonuclear leucocytes and macrophages. A transient elevation of intracellular Ca ion and O_2^- generation have been observed in neutrophils when the cells were treated by the peptides. Unlike mastoparan, the peptide does not affect on the GTP-binding protein but interact directly to FMLP receptor in neutrophils.

Novel neurotoxins in the spider, genus *Nephila*. spider toxin obtained from the venom of the spider belonging to the genus *Nephila* has been recognized as a new neurotoxin(JSTX, NSTX) which supresses irreversively the excitatory postsynaptic potential and the glutamate potential in the lobster neuromuscular junction with high degree of specificity. We purified the active principles from the venom of *Nephila clavata* and *N. maculata* and found that JSTX and NSTX composed of many kinds of the structurally similar toxic principles. Some of these toxic principles identified chemically as their common structure composed of a 2,4-dihydroxyphenylacetyl asparaginyl cadaverinocarboxyethylamino-derivative[3]. Some of these toxins and the related compounds were chemically synthesyzed and the structure-activity relationship was investigated. Another series of the toxic principle were also characterized from the venom of *N. clavata*.

JSTX was made radioactive by labelling with iodine-125. ^{125}I-JSTX irreversively blocked the EPSP of the lobster neuromuscular synapse in a similar manner as the natural toxin. Light microscope autoradiography of ^{125}I-JSTX treated muscle showed that radioactive spots were localized in the synaptic area along with the fine nerve branches. Electron microscopic analysis showed that these spots were coincided with the structure characteristic of the neuromuscular synapse. This finding gives morphological evidence that JSTX binds to the glutamate receptor-ion channel molecules.

1. Akizawa, T., Yasuhara, T., Kano, R. and Nakajima, T. Biomed. Res., 1985 6 437-441
2. Nakajima, T. In "Venoms of the Hymenoptera" T.Piek ed., 1986 pp.309-327 Academic Press
3. Aramaki, Y., Yasuhara, T., Higashijima, T., Yoshioka, M., Miwa, A., Kawai, N. and Nakajima, T. Proc. Japan Acad., 1986 62 Ser. B 359-362

Insect Peptide Hormone Biosynthesis

Michael O'Shea, Siegfried Hekimi and Marie-Francoise Schulz
Laboratoire de Neurobiologie, Université de Genève
20 rue Ecole-de-Médecine, CH-1211 Genève 4, Switzerland

Abstract:

We have studied the synthesis of the sequenced locust adipokinetic hormones AKH I and II (pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2 and pGlu-Leu-Asn-Phe-Ser-Thr-Gly-Trp-NH_2). These hormones are made in the corpus cardiacum (CC) and are important regulatory peptides which stimulate lipid metabolism during prolonged activity such as migratory flight. Two precursors, P1 and P2, of the AKHs have been identified by a variety of molecular, biochemical and immunochemical methods. Using *in vitro* tissue culture of the CC we have shown that these AKH precursors also produce two additional peptides we call AKH Precursor Related Peptides or APRP 1 and 2.

Protein sequencing has been used to determine the primary structures of the APRPs. Under reducing conditions the APRPs become smaller in molecular weight consistent with them being dimers linked by disulphide bridges. We believe APRP 1 is a homodimer of a 28 amino acid chain (α-chain) and APRP 2 is probably a heterodimer, also of 28 residue peptides (α and β). Messenger RNA extracted from the CC has been used to direct protein synthesis in an *in vitro* cell-free translation system (wheatgerm). This shows that CC mRNA is very efficiently translated primarily into only two polypeptides of about 6Kd. The simplicity of this pattern of protein synthesis from CC mRNA is consistent with our findings in the tissue culture system showing that P1 and P2 are primary products of the CC neurosecretory cells.

A λgt10 cDNA library has been constructed from the CC mRNA and screened with oligonucleotides based on the structure of the AKHs and APRPs. Positive clones are being analysed.

Developmental studies indicate that strong regulatory control is exerted on the synthesis of the precursors and its peptide products. The mechanism which underlies this control is under investigation as is the biological activity of the newly discovered AKH Precursor Related Peptides.

Spider Venoms as Probes for Insect Neurotransmission

Ian R. Duce

Department of Zoology, University of Nottingham, University Park, Nottingham NG7 2RD.

Venoms and toxins have provided a selection of powerful tools for studies of the nervous system. Historically these materials have played a prominent part in many breakthroughs in neurobiology and continue to be important in several recent developments involving the isolation and purification of receptors and ion channels. Natural products have also played a key role in the development of several groups of pesticides. In view of these observations it is likely that a group of animals, such as spiders, which are predominately insectivorous and which use venom to subdue their prey may provide a source of materials with potential as insecticides or tools for studying the insect nervous system.

In recent years we have studied the effects of venoms from several groups of spiders on insect nerve and nerve muscle preparations using a range of neurophysiological techniques. Initially this work was directed towards a search for compounds with activity at the L-glutamate receptor/ion channel complex of the insect. Venoms from several families of spider were screened for inhibition of the neurally evoked twitch contraction of the locust retractor unguis muscle. Apparent activity at the post-junctional site was confirmed by examining the inhibition of excitatory potentials induced by the ionophoresis of L-glutamate. Activity of this type was found in the venoms of *Argiope trifasciata* and *Araneus gemma*.[1] The active components were found to be low molecular weight compounds which we and two other groups have chemically characterised [2,3,4] and which are referred to as Argiotoxins. The Argiotoxins show chemical similarities to toxins JSTX and NSTX isolated from other orb-web weaving spiders [5]. These spider toxins all appear to act as antagonists of L-glutamate receptor/channel complexes.

Toxicological and pharmacological studies of spider venoms have concentrated largely on their effects on vertebrate preparations, however a spectrum of effects have been described on insect nervous tissue. In addition to the post synaptic effects described above presynaptic actions of venoms have been described in insects. The well known effect of black widow spider venom on vertebrate motor endplates is reflected in a massive increase in spontaneous release of neurotransmitter from locust neuromuscular junctions[6]. This effect is also produced by venom from other theridiid spiders such as *Steatoda grossa*, and *Steatoda bipunctata*. A presynaptic locus of action has also been implicated in the action of *Steatoda paykulliana* venom on central synapses in cockroaches[7].

Data obtained using whole milked venom or venom gland extracts may be misleading in that spider venoms are complex "cocktails"

of chemicals of widely differing molecular weight and type. Our studies with the venoms of several groups of spiders have suggested multiple sites of action may be involved in toxicity. Adams and colleagues[8] showed that *Agelenopsis aperta* venom contained toxins with pre- and post- synaptic actions. It is thus apparent that in order to produce materials of value for pharmacological or pesticidal application extensive purification and characterisation of spider venoms will be essential. However it is also clear that these materials are likely to be a valuable resource worthy of expliotation in the search for compounds with specificity for sites within the insect nervous system.

1. Bateman, A. Boden, P. Dell, A. Duce, I.R. Quicke, D.L.J. and Usherwood, P.N.R. Brain Res. 339 237-244 1985.

2. Budd, T.C. Clinton, P. Dell, A. Duce, I.R. Johnson, S.J. Quicke, D.L.J. Taylor, G.W. Usherwood, P.N.R. and Usoh, G. Brain Res. (in press).

3. Grishin, E.V. Volkova, T.M. Arseniev, A.S. Reshetova, O.S. Onosprienko, V.V. Magazanic, L.G. Antonov, S.M. and Fedorova, I.M. Bioorg. Chim. 12 1121-1124 (1986).

4. Adams. M.E. Carney, R.L. Enderlin, F.E. Fu, E.T. Jarema, M.A. Li, J.P. Miller, C.A. Schooley, D.A. Shapiro M.J. and Venema, V.J. Biochem. Biophys. Res. Comm. 148 (1987).

5. Aramaki, Y. Yashura, T. Higashyima,T. Yoshioka,M. Miwa, A., Kawai, N and Nakajima, T. Progr. Jpn. Acad.Ser. B. 62359-362 (1986).

6. Cull-Candy, S.G. Neal, H and Usherwood, P.N.R. Nature (Lond.) 241 353-354 (1973).

7. Tashmukhamedov, B.A. Usmanov,P.B. Kazakov, I. Kalikulov, D. Yukelson, L.Ya. Atakuziev, B.U. in *Toxins as Tools in Neurochemistry* Walter de Gruyter Berlin (1983).

8. Adams, M.E. Endelin,F.E. Cone, R.I. and Schooley, D.A. Proc. Int. Soc. Toxinology (1986).

EFFECTS OF SPIDER VENOMS ON THE VERTEBRATE NERVOUS SYSTEM

Hunter Jackson and Thomas N. Parks

Department of Anatomy, University of Utah Medical Center, Salt Lake City, Utah 84132

Spider venom research has for many years concentrated on venoms of spiders that are dangerous to man. As a consequence of that work, it has been known for some time that venoms of some of those spiders (e.g., black widows [*Latrodectus* spp.] and Sydney funnel web spiders [*Atrax* spp.]) contain toxins affecting the vertebrate nervous system. One such compound, a-latrotoxin, has found widespread application in neurobiology research by virtue of its effects on presynaptic transmitter release.

Interest in spider venoms on the part of the neuroscience community has greatly increased, however, since the discovery that certain orb-weaving spiders produce small toxins targeting the receptor-ion channel complexes associated with excitatory amino acid neurotransmitters. To appreciate that interest, one must understand that excitatory amino acid neurotransmitters, especially glutamate, appear to be the predominant excitatory transmitters in the vertebrate CNS. They have now been associated with such normal brain functions as learning and memory and a variety of neurological disorders including anoxic damage, Huntington's disease, and epilepsy. In 1982, Kawai and colleagues[1] reported that a toxin from the orb-weaving spider *Nephila clavata* produced potent and long-lasting suppression of excitatory amino acid transmission in the vertebrate brain. That finding, together with the fact that the insect prey of spiders employ glutamate as a transmitter at their neuromuscular junctions, provided the basis for investigating spider venoms as a new source of much needed excitatory amino acid antagonists. Although supply problems have limited research in this area, several venoms have now been shown to have excitatory amino acid antagonist activity.

More surprisingly and just as importantly from the perspective of a vertebrate neurobiologist is the finding that certain spider venoms also contain calcium channel antagonists. Of the four such toxins reported to date, three are found in the venoms of spiders belonging to the family Agelenidae (funnel-web spiders) while one is produced by a spider from the family Plectreuridae. Two of these four toxins, (found in the venom of the *Agelenopsis aperta* spider) affect the vertebrate CNS, whereas the other two have not affected vertebrate preparations tested thus far. The two calcium antagonists from the *Agelenopsis* spider have extremely unusual properties that make them of considerable potential value for basic research. In addition, at least one of the two has been shown to have anti-convulsant properties which serve to illustrate the potential clinical applications of calcium antagonist toxins or their derivatives.

[1] Kawai, N., Niwa, A., and Abe, T. Biomed. Res. 1982, 3 353-355.

"PURIFICATION OF AN INHIBITOR OF BRAIN SYNAPTIC MEMBRANE GLUTAMATE BINDING SITES FROM THE VENOM OF THE SPIDER ARANEUS GEMMA. E. K. Michaelis, Cntr. Biomed. Res., Univ. of Kansas, Lawrence, KS, 66045, USA.

Spider venoms from Araneus gemma, Neoscona arabesca and Argiope aurantia were screened for inhibitors of glutamate binding to its receptor sites in brain synaptic membranes. All venoms inhibited glutamate binding to these sites and the venom from A. gemma was the most active. This venom contains high concentrations of proteins and L-glutamic acid and low levels of catecholamines and other amino acids. Pressure ejection of glutamate into the caudate nucleus of rat brain caused enhancement of K^+ efflux from neurons. Ejection of venom inhibited glutamate-stimulated K^+ efflux, which was indicative of the presence of an inhibitor of glutamate receptors. Consistent separation of venom constituents has been obtained by reverse phase (RP) HPLC and two peaks of inhibition of glutamate binding were detected. One represented free glutamate whereas the other contained small amounts of glutamate together with other unidentified constituents that were enriched in secondary amines. All free glutamate was removed by anion exchange chromatography and material that inhibited glutamate binding activity was isolated by cation exchange chromatography followed by RP HPLC. Insufficient amounts of this material have been obtained and final identification of the chemical structure has not yet been achieved, although, UV spectral analyses indicated a chromophore with characteristics similar to those of either hydroxyphenyl or hydroxyindole groups. New isolation procedures and final identification of active constituents are being pursued.

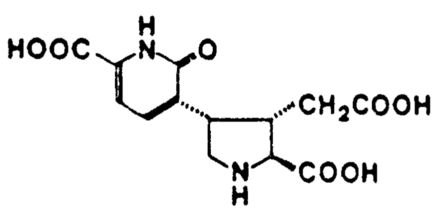
NOVEL EXCITATORY AMINO ACID RELATED COMPOUNDS OF NATURAL ORIGIN

H. SHINOZAKI

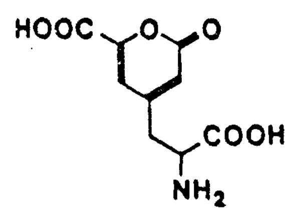
The Tokyo Metropolitan Institute of Medical Science, Tokyo 113, Japan

Pharmaco.ogy began when man first used a plant extract to relieve the symptoms of disease, and naturally occuring substances have played an important role in the neuropharmacological research. In the course of our pharmacological studies on excitatory amino acids, quisqualate and kainate were found to have marked excitatory actions in both invertebrates and vertebrates, and theanine, matrine and tuberostemonine demonstrated glutamate inhibitory actions at the crayfish neuromuscular junction (NMJ). They are all naturally occurring substances. We report here on electrophysiological actions of acromelic acid and stizolobic acid, which are novel amino acids of natural origin, in order to encourage the utilization of these new and potentially valuable compounds.

Acromelic acid is one of kainoids (kainate related compounds) isolated from a poisonous mushroom, and markedly depolarizes crayfish opener muscle fibers in a dose-dependent manner, and its potency is about 10 times as potent as that of domoic acid (acromelic acid is about 100 times more potent



Acromelic acid A



Stizolobic acid

than kainic acid). Brief iontophoretic application of acromelate produced a depolarization of the crayfish muscle, which lasted longer than the glutamate potential, and the time constant of its decay phase was more than 2 times larger than that of the glutamate potential. This is in contrast to kainic and domoic acid which are not able to produce a fast and large depolarization even when large amounts were iontophoretically applied at this junction. Moreover, acromelic acid markedly potentiated the glutamate response but depressed the quisqualate response, in spite of the fact that both glutamate and quisqualate act on the common receptor at the crayfish NMJ. Acromelic acid induced spike discharges from rat cortical neurones and caused a significant depolarization of motoneurones in the new-born rat spinal cord.

Stizolobic acid and stizolobinic acid, amino acids isolated from a plant, reduced responses to quisqualate and glutamate in a competitive manner at the crayfish NMJ, without affecting responses to GABA and acromelic acid. Stizolobic acid neither affected the resting membrane potential nor the input resistance of the crayfish opener muscle. The amplitude of excitatory junctional potentials was decreased by stizolobic acid in a concentration dependent manner. The blocking action of stizolobic acid was not use-dependent at the crayfish NMJ. Stizolobinic acid was about 5 times less potent than stizolobic acid. On the other hand, in the mammalian CNS, stizolobic acid caused a depolarizing response contrary to our expectation. Iontophoretic application of stizolobic acid to the rat cerebral cortical neurones induced significant spike discharges. The depolarizing response of the new-born rat spinal cord ventral roots caused by stizolobic acid was not affected by the existence of Mg^{2+} ions or APV, suggesting that stizolobic acid does not bind to the NMDA-preferred receptor in the rat spinal cord.

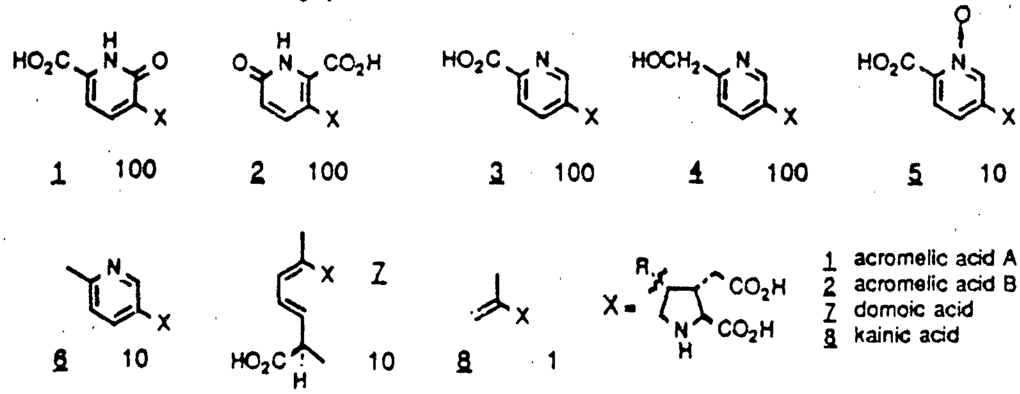
ACROMELIC ACID A AND B, VERY POTENT EXCITATORY AMINO ACIDS FROM A POISONOUS MUSHROOM

Haruhisa Shirahama

Faculty of Science, Hokkaido University, Sapporo, 060 Japan

Extremely potent neuroexcitatory amino acids, acromelic acid A and B, were isolated from a poisonous mushroom Clitocybe acromelalga Ichimura. Because of small quantities (110μg and 40μg respectively), MS, IR, ^{13}C-NMR could not be observed and the structure were deduced from ^{1}H-NMR and UV spectra and biogenesis. In order to confirm the structures and observe physiological activity, both acids were synthesized from kainic acid. The structures were consequently confirmed and absolute configurations were also determined. Acromelic acid A was represented as (2S, 3S, 4S)-3-carboxymethyl-4-(6-carboxy-2-pyridon-3-yl) proline (1) and B as (2S, 3S, 4S)-3-carboxymethyl-4-(6-carboxy-2-pyridon-5-yl)proline (2). Examination of the action of acromelic acids at a crayfish neuromuscular junction revealed that both of the acids yielded the most potent depolarization so far (100 times more potent than kainic acid). Neuroexcitatory action of the several derivatives of acromelic acid was observed and it was suggested that a pyridine ring equipped at C(4) of proline effected the strength of the action.

Relative strength of potenciation of excitatory responses to glutamate induced by acromelic acid A, B and their derivatives is roughly indicated.



TRIOXABICYCLOOCTANES AS PROBES FOR THE CONVULSANT SITE OF THE GABA-GATED CHLORIDE CHANNEL

John E. Casida[1], Russell A. Nicholson[2] and Christopher J. Palmer[1]

[1]Pesticide Chemistry and Toxicology Laboratory, Department of Entomological Sciences, University of California, Berkeley, California 94720, U.S.A.

[2]Wellcome Research Laboratories, Berkhamsted Hertfordshire, U.K.

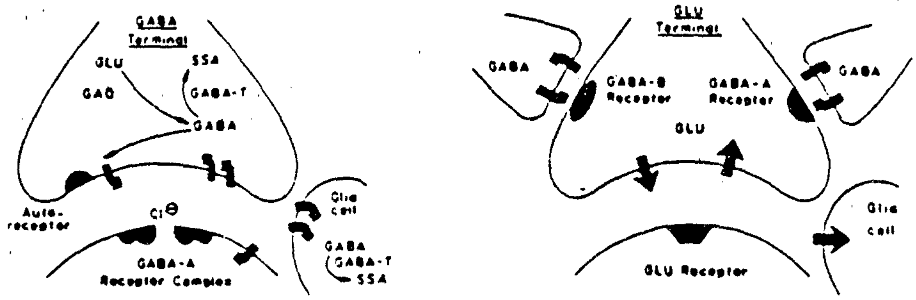
Bicyclophosphorus esters [$R\text{-}C(CH_2O)_3P{=}X$; X=O or S] and bicycloorthocarboxylates [$R\text{-}C(CH_2O)_3C\text{-}R'$] with suitable R and R' substituents [e.g., R = $(CH_3)_3C$ and R' = substituted-phenyl] are potent $GABA_A$ receptor antagonists and inhibitors of GABA-stimulated chloride flux. Some of these 2,6,7-trioxabicyclo[2.2.2]octanes also have significant levels of insecticidal activity. Radioligands from this class are useful in binding assays with nerve membranes to probe the convulsant site(s) of the GABA-gated chloride channel in insects and vertebrates. Many compounds act *in vitro* at 0.1 to 10 nanomolar and *in vivo* at 0.01 to 1 mg/kg. Selective toxicity is potentially conferred by species differences in the nature of the target sites and in metabolic activation or detoxification. The polychlorocycloalkane insecticides also act at the same or a similar binding site.

MOLECULAR PHARMACOLOGY OF GABA SYNAPTIC MECHANISMS

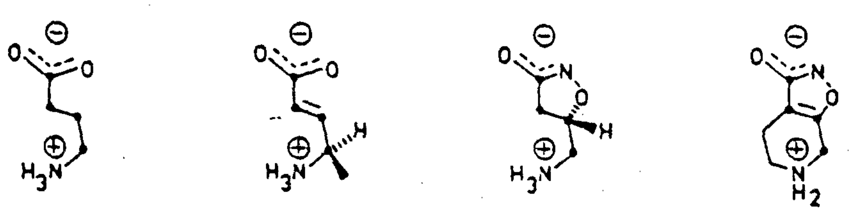
P. Krogsgaard-Larsen, L. Nielsen, J.S. Jensen, E. Falch,
L. Brehm and F.S. Jørgensen

The Royal Danish School of Pharmacy, Department of Chemistry BC,
2 Universitetsparken, DK-2100 Copenhagen, Denmark

The neutral amino acid 4-aminobutyric acid (GABA) is an inhibitory neurotransmitter in the mammalian CNS. Approximately 30-35% of all central neurones are utilizing GABA as a neurotransmitter, keeping virtually all neurones in the CNS under GABA control. These effects are mediated by at least two classes of distinctly different receptors (GABA-A and -B receptors), and there is some evidence of the existence of GABA autoreceptors. GABA is transported by multiple transport (uptake) systems located in neurones and glial cells, and GABA is metabolized by the enzyme GABA-T. There is an increasing pharmacological and therapeutic interest in agents capable of stimulating specifically GABA synaptic mechanisms, agonists at distinct receptor subtypes and inhibitors of glial GABA uptake or GABA-T being of primary interest.



The molecule of GABA is characterized in a high degree of conformational mobility allowing GABA to adopt a variety of different conformations. This molecular property of GABA may be essential to its complex physiological role. A prerequisite for the development of specific GABAergic drugs on a rational basis is information about the "biologically active" conformations of GABA. Via design of model compounds as exemplified below, in which stereochemical, conformational and electronic parameters have been systematically varied, compounds with specific and very potent actions at different GABA synaptic mechanisms have been developed.



GABA (R)-(+)-Me-t-ACA (S)-(+)-DHM THIP

The structural parameters of these model compounds have been mapped out using X-ray, computer molecular modelling and spectroscopic techniques. Adjustment of physicochemical parameters of pertinent model compounds, such as the GABA-A agonist THIP, have led to specific zwitterionic GABAergic agents capable of penetrating the blood-brain barrier.

These studies are consistent with the view that GABA adopts distinct "biologically active" conformations at different synaptic mechanisms, which exhibit dissimilar or, in some cases, opposite stereochemical requirements. Elucidation of these aspects has made pharmacological and clinical studies of the consequences of selective manipulation of different GABA synaptic mechansisms possible.

STRUCTURE-ACTIVITY RELATIONSHIPS OF (+)ANATOXIN-a DERIVATIVES AND ENANTIOMERS OF NICOTINE ON THE PERIPHERAL AND CENTRAL NICOTINIC ACETYLCHOLINE RECEPTOR SUBTYPES.

Y. Aracava, R. Rosental, K.L. Swanson, H. Rapoport & E.X. Albuquerque. Dept. Pharmacol. Exp. Therap., Univ. of Maryland Sch of Med., Baltimore, MD 21201.

The stereospecificity and potency of semi-rigid agonists (+)-anatoxin-a (AnTX) and (-)-nicotine (NIC) were exploited to study the function of nicotinic acetylcholine receptor ion channel complexes (nAChR) in central and peripheral nervous systems using patch clamp techniques. Channel currents induced by (+)-AnTX and (-) or (+)NIC on skeletal muscle fibres from the frog were similar to those activated by acetylcholine (ACh) although bursts were divided into a few brief openings. (+)-AnTX appears to act purely as an agonist or desensitizing agent by binding to the α-bungarotoxin (α-BGT) site. The allosteric site has less (or different, see below) stereospecificity; it was therefore possible to demonstrate that (-) and (+)-NIC also have noncompetitive blocking actions.

The nAChRs of the rat brain, i.e., [^{3}H]NIC and ^{125}I-α-BGT binding sites, have also been differentiated by the selective binding of (+)-AnTX to the high affinity NIC binding site. Using (+)-AnTX, channel activity was recorded on neurons cultured from neonatal rat hippocampi and basal ganglia. The channels activated by ACh were similar, although more brief. (+)-AnTX is a poor ligand for the muscarinic binding site of rat brain (Proc. Natl. Acad. Sci. USA, 78: 4639, 1981). This demonstrates the presence of functional central nAChRs (FEBS Lett. 222: 63, 1987).

(+)-AnTX analogs with modifications of the amine moiety and the ketone side chain were tested for actions on the nAChR of skeletal muscle and *Torpedo* electroplaque. These similar receptor types had 2 sites which were sensitive to the analogs: both the agonist (α-BGT) site and an allosteric site. Interaction with the latter site caused noncompetitive inhibition and was characterised by histrionicotoxin (HTX) binding. Complementary biochemical and electrophysiological techniques correlated 1) the binding to α-BGT site with contracture potency and stimulation of HTX binding and 2) the inhibition of muscle twitch with antagonism of HTX binding. Patch clamp studies demonstrated the microscopic kinetics of changes in ion channel conformation which were induced by these derivatives. In the case of noncompetitive inhibition, the kinetics were compared to those described by the sequential open channel blocking model.

SUPPORTED BY: NIH Grant NS23050

THE BIOCHEMICAL CHARACTERISATION OF INSECT GABA RECEPTORS

GEORGE G. LUNT, Department of Biochemistry, The University of Bath, Bath, England, BA2 7AY

The $GABA_A$ receptor complex of mammalian brain is a 4 subunit protein that has distinct but interacting binding sites for several groups of ligands. GABA binding in vivo results in the opening of the receptor's integral Cl^- channel and this effect can be modulated by benzodiazepines, barbiturates, cage convulsants and picrotoxinin. Additionally several insecticidal agents also affect receptor function; of particular interest are the avermectins and the cyclodienes. Thus it is assumed that insect GABA receptors may resemble their mammalian counterparts and may represent attractive targets for insect specific neurotoxic agents.

Several laboratories have demonstrated in insects the presen of binding sites that resemble in some respects those of mammalian brain $GABA_A$ receptors. In our own laboratory we have shown that in locust ganglia there are binding sites for GABA, benzodiazepines, barbiturates, cage convulsants and picrotoxinin that interact in a modulatory manner. In several respects these sites differ from the corresponding site on mammalian $GABA_A$ receptors. Thus the GABA site is insensitive to bicuculline, the benzodiazepine site shows a Ca^{++} dependence and a different pharmacology and the cage convulsant site shows complex binding kinetics. In the case of the benzodiazepine binding site photoaffinity labelling reveals a subunit with a molecular weight distinct from that of mammalian $GABA_A$ receptor αsubunit. The relationship between the cage convulsant site and the picrotoxinin site also appears to be different in that TBPS binding is unaffected by picrotoxinin.

These findings lend support to the view that insect GABA receptor complexes may represent potential targets for insect specific control agents.

TRANSMITTER RECEPTORS ON INSECT NEURONAL SOMATA: GABA-ERGIC AND CHOLINERGIC PHARMACOLOGY.

Jack A. Benson

R & D Plant Protection, Agricultural Division, CIBA-GEIGY Ltd., CH-4002 Basel, Switzerland.

How similar is the pharmacology of insect receptor subtypes to that of mammalian subtypes in the same and different receptor families? To what extent can chemicals used to define receptor subtypes in the mammalian nervous system (classical diagnostic compounds) be relied on to characterise receptors and ion channels in insects? To answer these questions, we have established pharmacological profiles for the GABAergic and cholinergic receptors on the somata of neurones from the thoracic ganglia of the locust, Locusta migratoria.

Following mechanical separation, these somata remain viable for hours under voltage clamp conditions. When exposed to brief, pressure micro-applications of GABA, they respond with a current which reverses at -55 to -65 mV and is carried by Cl^- ions. Acetylcholine evokes two pharmacologically separable responses, a "nicotinic" ACh1 response and a "muscarinic" ACh2 response. The GABA receptors belong to the $GABA_A$ receptor family but exhibit clear pharmacological differences from the corresponding vertebrate receptors. For example, they are insensitive to bicuculline. The ACh1 receptors also differ from their vertebrate counterparts, sharing characteristics of both muscular and neuronal nicotinic acetylcholine receptors as well as possessing unique properties.

The activity of a number of classical diagnostic compounds on the ACh1 receptors suggests that the specificity of such compounds is not always the same in insects as in other animal groups. Tetraethylammonium (TEA) is often used as a selective blocker of specific K^+ channels although it is also a ganglionic cholinergic blocker in mammals. Strychnine is recognised as a selective glycine antagonist although it also has been associated with cholinergic effects. Picrotoxin (PTX) is a selective blocker of Cl^- channels, especially those associated with $GABA_A$ receptors, and bicuculline is the diagnostic antagonist of $GABA_A$ receptors in vertebrates. All four of these compounds act as blockers of the response mediated by ACh1 receptors in the locust neuronal somata. In the case of PTX, its action against the GABA response occurs at a concentration several orders of magnitude below the effective dose against the cholinergic response. However, the other three compounds are potent in or below the concentration ranges within which their "classical" vertebrate effects occur.

ACTION OF TOXICANTS ON GABA AND GLUTAMATE RECEPTORS

Mohyee Eldefrawi & Amira Eldefrawi

Department of Pharmacology & Experimental Therapeutics
University of Maryland, School of Medicine
Baltimore, Maryland 21201, U.S.A.

GABA and glutamate receptors of mammalian brain and insect muscles are identified by radioactive ligand binding and ion flux measurements. Interactions of drugs and toxicants with these receptors are inferred from their effects on either ligand binding and/or receptor-regulated ion fluxes.

The GABA receptor is a primary target for the toxic action of many insecticides which inhibit its function by binding in or close to its chloride channel. These include polychlorocycloalkane compounds. There is good correlation between the inhibition by cyclodienes and γ-hexachlorocyclohexanes of the binding of [^{35}S]tert-butylbicyclophosphorothionate (TBPS) and their inhibition of GABA-regulated chloride transport. Type II pyrethroids also inhibit GABA-regulated Cl^- transport stereospecifically but with less potency than the cyclodienes. These pyrethroids bind with similar affinities to a mitochondrial protein associated with Ca^{2+} transport and labeled by [^3H]RO5-4864.

Although several organophosphate anticholinesterases and flame retardants inhibit the GABA receptor, there is no correlation between their effects on GABA receptor function and their anticholinesterase activities or delayed neuro- toxicities. Inhibition of GABA receptor be responsible in part for the toxic action of several natural mycotoxins. Tremorgenic mycotoxins that cause animal staggers and the antihelminthic-insecticidal Avermectin B_{1a} are also inhibitors of the GABA receptor.

In collaboration with Professors K. Nakanishi and P.N.R. Usherwood, philanthotoxin was isolated from the venom of the digger wasp *Philanthus triangulum*, its structure was identified and was synthesized. It inhibits reversibly glutamatergic transmission in insect muscle. It has no effect on the binding of [^3H]glutamate to rat brain or invertebrate muscle membranes. On the other hand, it inhibits noncompetitively the glutamate-enhanced binding of [^3H]MK-801 to rat brain N-methyl-D-aspartate receptor.

SECOND MESSENGER SYSTEMS IN INSECTS: AN INTRODUCTION

Peter D. Evans

AFRC Unit, Dept. of Zoology, University of Cambridge, Cambridge CB2 3EJ

Second messenger systems are used to link the activation of membrane bound receptors by external stimuli, such as hormones and neurotransmitters, to intracellular response mechanisms. Responses mediated by second messenger activation usually far outlast the period of receptor activation enabling a minimum amount of hormone or transmitter to produce a long-lasting effect. In addition, weak signals can be amplified many fold by the use of a second messenger system, so that the system can be made very sensitive to small changes in the concentration of the agonist. Further, second messengers enable responses to be elicited in regions of a cell at a distance from the site of agonist receptor interactions. Since there are many steps in the second messenger mediated pathways between the binding of the agonist to a receptor and the actual response system, such mechanisms are ideally suited to be modulated by other events, so that responses may be amplified or inhibited depending perhaps on the arousal state of the insect.

Currently there is much interest in a considerable array of different second messenger systems. However, in insects most of the work in this field has been restricted to a consideration of only two subclasses. namely those that use cyclic nucleotides and those that are mediated by the enzymatic breakdown of phosphoinositoids. For example, a considerable amount of

evidence suggests that many of the actions of octopamine on insect tissues, such as skeletal muscle, light organs, fat cells, blood cells, oviducts, are all mediated via an activation of adenylate cyclase activity which raises intracellular levels of cyclic AMP. In addition evidence is accumulating for a role for changes in cyclic GMP levels in the mediation of the actions of eclosion hormone. The inositol phosphate pathway has been shown to mediate the actions of a number of different receptors in insect tissues. These include one class of 5-HT receptor on the blowfly salivary gland and muscarinic cholinergic receptors in the insect central nervous system. In addition it may mediate some of the actions of some neuropeptides in insect tissues.

Recent information suggests that the functional significance of changes in the levels of second messengers in insect tissues must be considered in relation to a number of other factors, including, physiological concentration range of the agonist, age of the tissue, compartmentation of response within a tissue, and availability of substrates. Some of these factors will be discussed.

Effects of Pyrethroids on Neural Protein Kinases and Phosphatases

Fumio Matsumura
Dept. of Environmental Toxicology and
Toxic Substances Program, LEHR Facility
University of California, Davis, CA 95616 USA

Various pyrethroids have been shown to interfer with normal functions of sodium channels and neurotransmitter release mechanisms. However, the biochemical mechanisms by which these chemicals cause such a variety of neural effects has been a mystery. Previously our research group has found that these chemicals along with some chlorinated hydrocarbon insecticides (e.g. DDT) inhibit the processes involved in ATP-hydrolysis, particularly those which exhibit high calcium sensitive ties. While some of those processes represent neural ATPases, we have recently shown that a significant portion of the above ATP hydrolyzing processes are carried by various protein kinases and phosphatases. The nature of the proteins, phosphorylated with low concentrations of gamma-^{32}P-ATP (e.g. 10^{-9} - 10^{-6}M), were studied using SDS polyacrylamide gel-electrophoresis. In the case of the rat brain and the squid optic lobe synaptosomes, the predominant protein kinases were (1) Ca^{2+}-calmodulin dependent (=CCPK), (2) c-AMP dependent (=PKA) and (3) calcium-phospholipid dependent (=PKC) protein kinases. Deltamethrin, used as a typical type II pyrethroid, inhibited (1) at 10^{-8} - 10^{-6}M and to a lesser extent (3). Surprisingly it did stimulate (2). By using a purified PKA and histone, it was found that this pyrethroid stimulates its phosphorylation action on histone at a low (>10^{-13}M) concentration. In the absence of histone, PKA is known to phosphorylate its own regulatory subunit. This process was inhibited by deltamethrin at the same concentration range. Thus it appears that the action of deltamethrin is to release the catalytic subunit from the inhibitory interference from its regulatory subunit. Also using a purified calcium-calmodulin sensitive phosphatase (calcineurin), it was found that deltamethrin inhibits protein dephosphorylation processes. However, such actions required high concentrations (10^{-5} to 10^{-4}M) of the pyrethroid. Such diverse actions of pyrethroids on these enzymes are expected to cause a variety of changes in the functions of key protein which are regulated by phosporylation/dephosphorylation process. One of the key proteins studied was synapsin I of which roles in synaptic transmitter releasing processes are being examined.

COUPLING OF MUSCARINIC RECEPTORS TO SECOND MESSENGER SYSTEMS IN LOCUST GANGLIA.

Michael J. Duggan and *George G. Lunt.

Medical Research Council Molecular Neurobiology Unit, Cambridge and *Department of Biochemistry, University of Bath, Bath.

The muscarinic acetylcholine receptor (mAChR) has a fundamentally different mechanism of action from that of the nicotinic acetylcholine receptor (nAChR). The nAChR is well characterised in both insects and vertebrates as a ligand gated sodium channel. The vertebrate mAChR has no integral ion channel and produces its effects by mobilizing GTP-binding proteins. These GTP-binding proteins can influence a class of potassium channel and the activity of adenylate cyclase and phosphatidylinositol-diphosphate specific phosphodiesterase. These effects are mediated by mAChRs of different pharmacology which have recently been shown to be the products of several different genes.

In insects the presence of an mAChR has been inferred from the demonstration of binding of radiolabelled ligands to ganglionic homogenates. Observations of physiological effects of muscarinic pharmacology in the insects are few in number and their possible mechanism of action has not previously been investigated.

In this study on locust cerebral ganglia, radioligand binding experiments have shown some of the complex pharmacology found in vertebrate systems. This finding has been used to

indicate the presence of multiple subtypes of mAChR. Furthermore, cholinergic agonists have been shown both to decrease the rate of accumulation of cAMP and to increase the turnover of phosphatidyl inositol. These effects can be antagonised by compounds of a muscarinic pharmacology and this is indicative of mAChR-mediated regulation of second messenger metabolism, analogous to that seen in mammals.

OCTOPAMINE RECEPTORS: BIOCHEMISTRY AND MODE OF ACTION

James A. Nathanson

Department of Neurology, Harvard Medical School, and Neuropharmacology Research Laboratory, Mass. General Hospital, Boston, MA 02114

Octopamine is a physiologically significant neurotransmitter in insects, having both neurohumoral and transmitter actions. Because exogenously applied octopaminergic agonists can disrupt insect behavior and interfere with feeding[1-3], the potential exists for the development of potent octopamine analogs which would have pesticidal or pestistatic toxicity for insects. Because membrane receptors for octopamine appear to be present primarily in invertebrates, such analogs would have reduced toxicity for mammals and other vertebrates. Recent experiments in our laboratory have been directed toward developing techniques which will lead to a greater understanding of the biochemistry of octopamine receptors (particularly those associated with the activation of adenylate cyclase) and to a better knowledge of how these receptors may vary among different insect species. In particular, we have been developing a series of octopamine receptor probes to characterize and isolate solubilized octopamine receptor proteins.

Recently, we have synthesized a new octopamine agonist, NC-5Z, which is more than 100 times as potent as octopamine, itself, in activating adenylate cyclase and is the most potent octopamine agonist yet described in the firefly light organ system. Under conditions of reversible binding, NC-5Z is a complete agonist whose activation can be inhibited by known antagonists of octopamine-sensitive adenylate

cyclase. NC-5Z is also very effective in activating adenylate cyclase in cockroach nerve cord and in Manduca sexta nerve cord, where it appears to be able to differentiate among two adenylate cyclase-associated receptor subtypes. The compound exerts antifeeding activity in Manduca sexta larvae and light emitting activity in the firefly light organ.

NC-5Z is not only potent but has been designed, under certain conditions, to bind irreversibly to octopamine receptors and to remain active in its covalently-bound state. We have developed a synthetic scheme for radioactively labeling this compound and have begun to show labeling, both autoradiographically (to insect tissues) and to solubilized octopamine receptor proteins. NC-5Z and related compounds should be of considerable use in investigating the interspecies distribution and molecular pharmacology of octopamine receptor subtypes, their histochemical localization, their coupling to adenylate cyclase, and their biochemical characteristics.

1. Hollingworth, R., Lund, A. In "Insecticide Mode of Action", Coats J.R., Ed., Academic: New York, 1982, pp. 189-227.
2. Nathanson, J.A. Proc. Natl. Acad. Sci. USA 1985, 82 599-603.
3. Nathanson, J.A. Molec. Pharmacol. 1985, 28 254-268.
4. Nathanson, J.A. In "Toxicants Affecting GABA, Octopamine, and Other Neuroreceptors in Invertebrates", Green, M., Hollingworth, R., Hedin, P., Eds., Am. Chem. Soc.: Wash., 1987, pp. 154-161.

OCTOPAMINE- AND DOPAMINE-SENSITIVE RECEPTORS AND CYCLIC AMP PRODUCTION IN INSECTS

Roger G.H. Downer

Department of Biology, University of Waterloo, Waterloo, Ontario, Canada, N2L 3G1

An adenylate cyclase complex that is GTP-dependent and sensitive to Mg^{++}, NaF and forskolin has been identified in several insect tissues. Incubation of membrane preparations from cockroach brain and nerve cord in the presence of 1×10^{-6} M octopamine or dopamine results in elevated levels of cyclic AMP and additivity studies indicate that distinct membrane receptors are involved in mediating the responses to the two monoamines. Octopamine, but not dopamine, also elevates cyclic AMP levels in preparations from cockroach haemocytes and locust nerve cord.

A variety of potential agonists and antagonists were tested to provide pharmacological characterisations of the octopamine and dopamine receptors. The octopamine-mediated stimulation of cyclic AMP production is mimicked by synephrine, tyramine, naphazoline, clonidine and several formamidine pesticides whereas the response is inhibited by mianserin, promethazine, phentolamine, gramine, cis-flupenthixol, cyproheptadine and dibenamine. However, differences in the pharmacology of the octopamine-mediated response between different tissues confirm earlier suggestions that there are several types of octopamine receptor[1]. Studies on dopamine-mediated elevation of cyclic AMP production indicate an agonistic response with the selective

D_2-agonist LY-171555 and no stimulation with the selective D_1-agonist, SKF-38393. However, studies with antagonists indicate moderate inhibition with both the selective D_1-antagonist SCH-23390 and the D_2-antagonist, spiperone. Thus, the data suggest that the insect dopamine receptor that is coupled to adenylate cyclase is pharmacologically distinct from dopamine receptors that have been described for vertebrate tissues.

Preliminary evidence will also be presented to suggest that some octopamine effects involve increases in intracellular calcium concentration and that octopamine-mediated cyclic AMP production may be modulated by protein kinase C.

1. Evans, P.D. J. Physiol. 1981 318 99-122.

MOLECULAR AND CELLULAR APPROACHES TO NEUROTOXICOLOGY: PAST, PRESENT AND FUTURE

Toshio Narahashi

Department of Pharmacology, Northwestern University Medical School, Chicago, Illinois 60611, U.S.A.

The mechanism of action of toxic substances on the nervous system has been studied for many years, yet it was not until 1980s that approaches and techniques were developed to allow us to study the toxic action at the molecular and cellular levels. The present paper is concerned mainly with neurotoxicology of insecticides, and represents some highlights of the study performed in our laboratories during the past 38 years. Emphasis is placed on the rationale of development of the study rather than mere historic aspects or technical details.

One of the earliest electrophysiological studies of insecticidal actions was performed in 1942 by Lowenstein[1] who discovered massive discharges in the insect nervous system intoxicated with pyrethrum extract. Similar stimulating actions were observed with other insecticides including DDT, lindane, dieldrin and organophosphates[2]. However, these studies remained totally phenomenological. A quantum leap was made toward the mechanism of action when intracellular microelectrode techniques were applied to cockroach giant axons. It was shown that repetitive after-discharges evoked by a single stimulus in the DDT- and pyrethroid-poisoned axon were induced by an increase in depolarizing after-potential that reached the threshold for generation of action potential[3,4].

The ionic mechanism underlying the increase in depolarizing after-potential was disclosed by using voltage clamp techniques which represented another quantum jump. The sodium channel current was shown to be prolonged by DDT and pyrethroids, causing an increase in depolarizing after-potential[5,6], which in turn would evoke repetitive discharges. However, since the sodium current thus recorded originated from a large number of sodium channels, we did not know how individual channels were affected. The breakthrough for this problem was made by adopting patch clamp techniques originally developed by Neher and Sakmann[7]. Individual sodium channels were kept open for only 1-2 msec under normal conditions, but the open time was prolonged by various pyrethroids to as long as several hundred milliseconds to several seconds[8,9]. Prolonged openings would cause a prolonged whole cell sodium current, and account for hyperactivity of the nervous system and animal.

Our knowledge was further advanced by measurements of ion fluxes and receptor binding. Although these studies will not be the major subject of discussion in the present paper, suffice it to say that some of the studies dealing with Na^+ fluxes across the membrane provided support to the notion of the pyrethroid-induced prolongation of sodium channel opening, while some others proposed a new hypothesis calling for the interaction of type II (α-cyano containing) pyrethroids with GABA receptor-channel complex as the basis for toxicity[10]. However, deltamethrin was much less potent on the GABA system than on the sodium channel[11,12], and had little or no effect on the specific binding of [^{3}H]dihydropicrotoxinin, a ligand for the GABA-activated channel[13]. More direct demonstration against the GABA hypothesis has recently been made by patch clamp experiments using the primary cultured neurons isolated from the rat dorsal root ganglion[14]. Application of 10 μM deltamethrin caused no change in the GABA-induced chloride current while increasing and prolonging the sodium channel current recorded from the same neuron in a manner characteristic of type II pyrethroids. Therefore, the GABA receptor-channel complex appears to play

little or no role in the toxic action of deltamethrin.

It has been known for a long time that cyclodienes and lindane stimulate synaptic transmission[2]. Ligand binding and Cl^- flux measurements indicated that these insecticides blocked the GABA receptor-channel complex[12,15,16]. The validity of this hypothesis has recently been demonstrated for lindane using the patch clamp technique[14]. The rat dorsal root ganglion neuron responded to GABA by generating a desensitizing (inactivating) and a non-desensitizing (steady-state) chloride current, and the former component was completely blocked by lindane (γ-BHC) but not by α-BHC. Therefore, this blocking action appears to be one of the factors responsible for synaptic stimulation caused by lindane.

Whereas our current research deals with the action of insecticides at the single channel level, the future of this field will be directed toward the molecular mechanism. One of such studies will be concerned with the identification of the molecular components of subunits of the target receptor-channel complex with which insecticides interact. This will be followed by characterization of the mechanism involved in insecticide-receptor molecule interaction. Current rapid developments in molecular biology and cell biology should be incorporated into such studies of insecticides.

1. Lowenstein, O. Nature 1942 150 760-762

2. Narahashi, T. Advances in Insect Physiology, ed. J.W.L. Beament, J.E. Treherne and V.B. Wigglesworth, Academic, London, 1971 8 1-93

3. Narahashi, T. J. Cell. Comp. Physiol. 1962 59 61-65

4. Narahashi, T. and Yamasaki, T. J. Physiol. 1960 152 122-140

5. Narahashi. T. and Haas, H.G. Science 1967 157 1438-1440

6. Narahashi, T. and Anderson, N.C. Toxicol. Appl. Pharmacol. 1967 10 529-547

7. Neher, E. and Sakmann, B. Nature 1976 260 779-802

8. Yamamoto, D., Quandt, F.N. and Narahashi, T. Brain Res. 1983 274 344-349

9. Chinn, K. and Narahashi, T. J. Physiol. 1986 380 191-207

10. Lawrence, L.J. and Casida, J.E. Science 1983 221 1399-1401

11. Chalmers, A.E., Miller, T.A. and Olsen, R.W. Neuropharmacology and Pesticide Action (Neurotox '85 Abstr.) 1985 41-42

12. Bloomquist, J.R. and Soderlund, D. Biochem. Biophys. Res. Commun. 1985 133 37-43

13. Matsumura, F. and Tanaka, K. Cellular and Molecular Neurotoxicology, ed. T. Narahashi, Raven, New York, 1984 225-240

14. Ogata N., Vogel, S.M. and Narahashi, T. Soc. Neurosci. Abstr. 1987 13 65

15. Abalis, I.M., Eldefrawi, M.E. and Eldefrawi, A.T. Pesticide Biochem. Physiol. 1986 24 95-102

16. Ghiasuddin, S.M. and Matsumura, F. Comp. Biochem. Physiol. 1982 73C 141-144

FUNCTIONAL COMPONENTS IN THE NERVOUS SYSTEM REVEALED BY INHIBITORY AND FACILITATORY TOXINS

J. Oliver Dolly
Department of Biochemistry, Imperial College, London SW7 2AY.

Neurotransmitter release and its modulation by voltage-activated K^+ channels have been studied using botulinum neurotoxin (BoNT) and dendrotoxin (DTX), specific and novel probes that respectively inhibit and facilitate secretion [reviewed in (1-3)].

BoNT represents a family of highly toxic di-chain proteins (M_r ~150,000) produced by *Clostridium botulinum* that cause botulism in humans and livestock, by specifically and irreversibly blocking Ca^{2} -dependent release of acetylcholine from peripheral nerve terminals. This involves targetting to cholinergic nerves by interaction of its heavier chain with ecto-acceptors, internalization and inactivation of a component of the release process. Recent electrophysiological recordings (4) in large cholinergic neurons showed that extra- or intra-cellular administration of BoNT type A or B blocked quantal release. Moreover, the toxin inhibited release from non-cholinergic neurons *provided* it was micro-injected into the cell. The two purified and individually-renatured chains of BoNT, which are non-toxic in mice, failed to affect the release of acetylcholine whether applied inside or outside the neurons. However, blockade of acetylcholine release was observed after intra-neuronal administration of *both* the heavy and light chains or when the latter was injected and the heavy chain bath-applied. In brain synaptosomes the release of several neurotransmitters is BoNT-susceptible and, likewise, secretion from exocrine cells can be prevented by the toxin. Thus, the presence of both chains is required *intra-cellularly* to inactivate an essential component of a ubiquitous secretion system. Investigations on the molecular nature of the action of this'universal' probe will be discussed in relation to the possible identity of its functionally important substrate.

DTX is one of a homologous group of basic, single-chain polypeptides (M_r ~7,000) purified from mamba snake venoms. It is a potent convulsant that facilitates transmitter release at both peripheral and central synapses. These actions appear to result from a selective blockade of at least two variants of fast-activating, voltage-dependent K^+ channels that are intimately involved in controlling nerve cell excitability and synaptic transmission (5,6). A K^+ current found in rat ganglia that inactivates slowly is most sensitive to DTX and 4-aminopyridine (although this is orders of magnitude less potent) whereas hippocampal CA_1 neurons display a faster inactivating K^+ conductance that is attenuated by larger concentrations of these agents; a third variety detected in superior cervical ganglion is virtually unaltered by DTX. Consistent with these widespread effects on K^+ channel sub-types, two or more populations of high affinity acceptors for ^{125}I-labelled DTX have been characterized in vertebrate brain. A minor species of this acceptor protein also binds β-bungarotoxin (a pre-synaptically active snake protein) and occurs predominantly on membrane of nerve terminals (7) whilst the more abundant DTX acceptor occurs also on somatic and axonal membranes (1). Although analysis of detergent-solubilised extracts of synaptic membranes shows that the DTX acceptor is a large glycoprotein (M_r ~400,000), a subunit of ~70,000 has been identified by cross-linking to DTX. Interestingly, this corresponds in size to protein 'isotypes' of a channel in *Drosophila* that have very recently been predicted from cDNAs identified, using a mutant *(Shaker)* with a defective A-type K^+ channel (8).

A comparison will be made between the postulated structure for the fly K^+ channel and the oligomeric/subunit properties established for the putative K^+ channel/DTX acceptors.

1. Dolly, J.O, Black, J.D., Black, A.R., Pelchen-Matthews, A. and Halliwell, J.V. Novel roles of neural acceptors for inhibitory and facilitatory toxins. *In:* Natural Toxins - Animal, Plant and Microbial, (J.B. Harris, ed.), pp. 237-264 (1986). Oxford University Press.

2. Dolly, J.O., Ashton, A.C., Evans, D.M., Richardson, P.J., Black, J.D. and Melling, J. Molecular action of botulinum neurotoxins: role of acceptors in targetting to cholinergic nerves and in the inhibition of the release of several transmitters. *In:* Cellular and Molecular Basis of Cholinergic Function, (M.J. Dowdall and J.N. Hawthorne, eds.), pp. 517-533 (1987). Ellis Horwood, Chichester.

3. Dolly, J.O., Stansfeld, C.E., Breeze, A., Pelchen-Matthews, A., Marsh, S.J. and Brown, D.A. Neuronal acceptor sub-types for dendrotoxin and their relation to K^+ channels. *In:* Neurotoxins and their Pharmacological Implications, (P. Jenner, ed.), pp. 81-96. Raven Press, New York.

4. Poulain, B., Tauc, L., Maisey, E.A., Wadsworth, J.D.F., Mohan, P.M. and Dolly, J.O. (1988) Neurotransmitter release is blocked intracellularly by botulinum neurotoxin and this requires uptake of both its polypeptides by a process mediated by the larger chain. Proc. Natl. Acad. Sci. USA (in press).

5. Halliwell, J.V., Othman, I.B., Pelchen-Matthews, A. and Dolly, J.O. (1986). Central action of dendrotoxin: selective reduction of a transient K conductance in hippocampus and binding to localized acceptors. Proc. Natl. Acad. Sci. USA 83, 493-497.

6. Stansfeld, C.E., Marsh, S.J., Parcej, D.N., Dolly, J.O. and Brown, D.A. (1987) Mast cell degranulating peptide and dendrotoxin selectively inhibit a fast-activating potassium current and bind to common neuronal proteins. Neurosci. 23, 893-902.

7. Pelchen-Matthews, A. and Dolly, J.O. (1988) Distribution of acceptors for β-bungarotoxin in the central nervous system of the rat. Brain Res. 441, 127-138.

8. Timpe, L.C., Schwarz, T.L., Tempel, B.L., Papazian, D.M., Jan, Y.N. and Jan, L.Y. (1988). Expression of functional potassium channels from *Shaker* cDNA in *Xenopus* oocytes. Nature 331, 143-145.

RECEPTORS FOR ACETYLCHOLINE IN THE NERVOUS SYSTEM OF INSECTS

H. BREER
University Stuttgart-Hohenheim
Institute of Zoophysiology
7000 STUTTGART 70, West Germany

The central nervous system of arthropods is highly cholinergic innervated; thus insect ganglia appear to be most appropriate for studying neuronal acetylcholine receptors (AChR). Using specific ligands, like α-bungarotoxin and quinuclidinylbenzilate, rather high concentrations of cholinergic binding sites have been detected in the head and thoracic ganglia of various insect species. In contrast to the preponderance of muscarinic receptors in the vertebrate brain, the nicotinic receptor type predominates in insects, whereas only a small portion displayed muscarinic properties. Nevertheless, both subtypes of the muscarinic receptor (M_1, M_2) have been discovered also in the insect nervous tissue. Subcellular fractionation experiments have revealed that the M_2-subtype appear to be predominantly located at the nerve terminals and to be involved in the feedback regulation of presynaptic activity, notably the evoked release of acetylcholine.

Attempts have been made to identify the nicotinic acetylcholine receptor from the nervous tissue of locust. A large complex protein, which binds α-toxin with high affinity has been solubilized from locust membrane preparations and purified via density gradient centrifugation and affinity chromatography to homogeneity. In microelectrophoretical analysis only a single polypeptide band could be detected, suggesting that the complex protein may be composed of identical or very simiiar polypeptides. When reconstituted in planar lipid bilayer, the native purified protein formed functional ion channels, activated by cholinergic agonists like acetylcholine, carbamylcholine or suberyldicholine and blocked by antagonists like d-tubocurarine, indicating that a functional acetylcholine receptor has been purified. The maximal conductance of the channel and its selectivity for cations are reminescent to the peripheral vertebrate receptor. Kinetic analysis of the channel gating have revealed that

multiple gating as well as bursting events appear at high agonist concentrations. Approaches to determine the cooperativity of channel activation showed that the nicotinic acetylcholine receptor from nerve cells of insects is apparently activated by one agonist molecule. The receptor protein has been localized in the neuropil of locust ganglia using monospecific antibodies in immunocytochemical experiments. Furthermore immunochemical analysis have shown that there are obviously significant molecular similarities between constituents of the neuronal insect receptor and the peripheral heterooligomeric vertebrate receptor; this was confirmed when the N-terminal amino acid sequence of an insect receptor polypeptide fragment was determined. As a first step towards an application of recombinant DNA-techniques, RNA preparations from the nervous tissue of young locusts were probed for receptor specific mRNA using reticulocytes and Xenopus oocytes as expression systems. It was found that oocytes microinjected with locust polyA$^+$ -RNA produced receptor polypeptides which bound α-toxins and could be precipitated by specific antibodies. Binding and ion flux studies have provided evidences that the expressed receptor polypeptides are inserted into the oocyte surface membrane and represent acetylcholine-gated ion channels, functional acetylcholine receptors.

GENETIC AND PHARMACOLOGICAL ANALYSES OF POTASSIUM CHANNELS IN *DROSOPHILA*.

C.-F. Wu, Dept. of Biology, Univ. of Iowa, Iowa City, Iowa 52242 U.S.A.

Several single-gene mutations in *Drosophila* are known to affect membrane currents, resulting in altered nerve and muscle excitability and abnormal behavior. Analysis of such mutants allows identification of the physiological roles of various currents and correlation of the distinct channel properties with specific structural domains altered by individual mutations.

Voltage clamp studies of *Drosophila* muscles revealed at least four K^+ currents that can be separated by differences in their properties as well as sensitivity to pharmacological blockade and mutational alteration. The two voltage-activated K^+ currents have been distinguished from each other by using *Shaker* (*Sh*) mutations which affect the fast K^+ current (I_A) but not the slow current (I_K). The *slowpoke* (*slo*) mutation provide a distinction between the two Ca^{++}-activated K^+ currents by eliminating the fast current (I_C) but not the slow current. The Ca^{++}-activated slow current and I_K exhibit similar kinetics but a clear pharmacological distinction exists between these two currents. Quinidine at a low concentration selectively blocks I_K without effects on other currents.

The *Sh* gene is thought to code for at least a component of the I_A channel. In the *Sh*^{5} allele we found that the voltage dependence and kinetics of I_A activation and inactivation were altered but the channel selectivity remained intact. In addition, *Sh*^{5} channels were much more sensitive to blockade by 4-AP. The results raise the possibility that the gating and 4-AP binding functions reside within the same structural domain of the channel. We further explored the number of *Sh* gene products and their stoichiometry within the channel by combing different alleles in heterozygotes and examined for evidence of interactions among dissimilar products. The results suggest the possibility of two different *Sh* products in the I_A channel. One class of *Sh* mutations resulted in a simple gene-dosage effect; the I_A in heterozygous combinations reflected additive contributions from two independent populations of I_A channels, indicating a product that function as a monomer within the channel. In contrast, a second class of *Sh* mutations caused drastic departures from the simple additive effect; the amplitude of I_A in heterozygotes was significantly smaller than that expected from gene dosage. This dominant effect can be explained by a second *Sh* product that is present in multimeric form within the channel.

Studies of single-channel events underlying these macroscopic currents have been initiated to examine the detailed mechanisms of mutational and pharmacological perturbations. The use of *slo* mutants has led to the positive correlation of a type of Ca^{++}-dependent channels with the macroscopic I_C. Further studies of the pharmacological profiles of different types of single-channel currents in normal and mutant preparations may extend our understanding of K^+ channel function and diversity.

ACETYLCHOLINE, GABA AND GLUTAMATE RECEPTOR CHANNELS IN CULTURED INSECT NEURONES.

by Yves Pichon
Département de Biophysique, Laboratoire de Neurobiologie Cellulaire et Moléculaire du C.N.R.S., F-91190 Gif-sur-Yvette (F)

Primary cultures of embryonic neurones can be prepared from embryos of the american cockroach, Periplaneta americana. These cultures provide some unique opportunities for pharmacological studies especially since they can be kept free of glial cell contamination. Under such conditions, (1) it is possible to control exactly the ionic environment, (2) the nerve membrane is directly accessible to putative transmitters or toxins, (3) membrane patches suitable for single channel analysis are easily formed without enzymatic treatment of the culture and kept for prolonged periods of time.

During the past three years, a large amount of informations has been gathered concerning the mode of action of the three main families of neurotransmitters on these cultured neurones. The experiments were carried-out at Thames Polytechnic, London, in David Beadles' Laboratory and in our Department at Gif. They are now in progress at Oxford Polytechnic. Most experiments were done using the patch-clamp technique in two different configurations: whole-cell-clamp and cell-attached. In the former case, the electrode contained 114 (or 140) mM KCl, 1.6 mM $MgCl_2$, 0.2 mM $CaCl_2$, 5 mM EGTA, 100 mM glucose and 10 mM Hepes Buffer at pH 7.2. In the latter, the electrode contained the same solution as the bath (i.e. 210 mM NaCl, 3.1 mM KCl, 10 mM $CaCl_2$ and 10 mM Hepes Buffer at pH 7.2.)

Acetylcholine (ACh) and its agonist Carbamylcholine (CCh) depolarize the neuronal membrane at micromolar concentrations. The reversal potential of this depolarization was found to approximate -10 mV. Spectrum analysis of the fluctuations induced by the two cholinergic agents revealed one (CCh) or two (ACh) lorentzian components. Single channel activity induced by the two cholinergic agonists consisted into short openings (0.2 to a few ms) with two conductance levels (around 20 and 50 pS). Careful statistical analysis of several thousands of single channel recordings suggest that these two conductance levels correspond to two different channels and not to substates of the same channel.

GABA and its agonist, muscimol, induce a current corresponding to the opening of chloride channels. Spectrum analysis of the noise induced by the application of the two inhibitory agonists revealed only one Lorentzian component with a corner frequency of around 15 Hz for GABA and 25 Hz for muscimol. The single channel conductance derived from the spectra lied around 16 pS for the two agonists. It has not yet been possible to confirm these figures using single channel data.

L-Glutamate has been applied in the presence and the absence of Concanavalin A (Con A). Some, but not all, neurones responded to pressure of iontophoretic application of L-glutamate onto the cell membrane, the response being a depolarization or a hyperpolarization. Unitary currents corresponding to the depolarizing response were recorded and analyzed. Micromolar concentrations of L-glutamate induce very short and usually complex single channel like activity. Analysis of long recordings of this activity strongly suggest that the channel possesses several (up to 4 or 5) substates at multiples of about 1 pA. Further experiments and analysis are needed to fully understand the complex kinetics of these channels.

The results will be discussed in the view of the most recent findings on other insect neurones and other neuronal preparations.

Molecular Biology of *Drosophila* Choline Acetyltransferase

Paul M. Salvaterra, Ph.D.
Division of Neurosciences
Beckman Research Institute of the City of Hope
1450 E. Duarte Rd.
Duarte, CA 91010 USA

Choline acetyltransferase (ChAT, EC2.3.1.6) is the enzyme responsible for the biosynthesis of the important central and peripheral neurotransmitter, acetylcholine. We have recently obtained detailed structural information about this protein by isolating and sequencing a cDNA clone from *Drosophila*. The results of these studies have led us to several interesting structural and functional conclusions regarding *Drosophila* ChAT and its evolutionary relationship to other macromolecules which interact with acetylcholine. A detailed comparison of the amino acid sequence of *Drosophila* ChAT with that of *Torpedo* acetylcholinesterase reveals a weak but significant homology between these two proteins. The homology is primarily confined to six peptide segments in each sequence, but is global when the peptides are ordered within each structure. These observations indicate that both proteins may have evolved from a common ancestral gene. We have also noted several weak amino acid analogous sequences when comparing ChAT with a neuronal acetylcholine receptor sequence. The analogous peptides are not extensive enough to propose a common origin for these two genes but may indicate regions of structural and functional convergence.

Another interesting structural aspect of ChAT involves the mechanism of protein translation initiation. We have succeeded in obtaining correct translation of our cDNA clone in several test systems including *Xenopus* oocytes, *E. coli*, and rabbit reticulocyte lysates. These results are unusual since our cDNA clone has no usual AUG initiation codon upstream from the known protein coding region. It seems likely that *Drosophila* ChAT may use a non-AUG codon to initiate protein translation. In an attempt to identify the likely initiation codon we have created several *in vitro* mutants and tested them for their ability to produce active enzyme of the proper size.

We have also recently completed studies which describe the pattern of ChAT mRNA and enzyme production during *Drosophila* development. The steady state mRNA levels appear to have a biphasic pattern of expression temporally preceding a similar pattern of expression for enzyme activity by several hours. The increasing phases of ChAT mRNA seem to correlate with development of cell-cell contacts within the nervous system. In temperature sensitive alleles of the ChAT gene, steady state mRNA levels seem to correlate well with the amount of active enzyme produced under various conditions. These results may indicate the presence of a positive feed back loop which is important for regulating ChAT expression *in vivo*.

PATCH-CLAMP ANALYSIS OF SINGLE CHLORIDE CHANNELS IN PRIMARY NEURONAL CULTURES OF DROSOPHILA.

Daisuke Yamamoto

Laboratory of Neurophysiology, Mitsubishi-Kasei Institute of Life Sciences, Machida, Tokyo 194, Japan

Although Drosophila melanogaster is potentially one of the best suited organisms for genetic and molecular biology approaches to the study of the nervous system, electrophysiological studies of individual neurons have been hampered by their extremely small diameter. The recent development of patch-clamp techniques has made it possible to study electrical properties of small, hitherto inaccessible cells. We have applied the gigaohm-seal patch clamp method to primary cultured Drosophila neurons, and here we report on a novel chloride channel present in the cell body membrane.

The channel has a conductance of 35pS with 145mM Cl^- in both sides of the membrane, and stays in the open state for tens of minutes with occasional interruptions by short closing transitions. The channel typically stays at either the fully closed or the fully open state. However, this rather simple behavior of the channel can readily be altered if the cytoplasmic side of the channel is exposed to the anionic buffers Hepes or Mops[1]. In the presence of Hepes or Mops, the open Cl^- channel seems to dwell in several discrete conductance levels, some of which are hardly detectable in the absence of these compounds. An increase in the buffer concentration increases the frequency of appearance of events with lower conductance at the expense of events with higher conductance, whereas the levels of the minimal and maximal step remain unchanged. The channel tends to stay

longer in the open states with higher conductances at more depolarized membrane potentials. These results are consistent with the following hypothesis: the single chloride channel is composed of multiple pores that are gated simultaneously, each of which is subject to an independent hit by a blocker (Hepes or Mops) molecule. As a concequence, the conductance of a partly blocked channel decreases in a stepwise fashion as the number of blocked pores increases.

To examine ionic selectivity of the channel, the cytoplasmic side of the inside-out patch membrane was exposed to solutions of different anionic composition. Total replacement of chloride with glutamate completely eliminates the inward current at a holding potential of -90mV. Large inward currents are observed with solutions containing NO_3^-, Br^-, or I^- in place of Cl^-. The channel is also permeable to F^-. The channel has the selectivity sequence $NO_3^-(1.97) > Br^-(1.12) \approx I^-(1.03) \approx Cl^-(1) > F(0.32) \gg$ glutamate(<0.02) as estimated by the permeability ratio based on the reversal potential measurement.

The chloride channel is blocked by SITS (4-acetoamido-4'-isothiocyanostilbene-2,2'-disulfonic acid), a classic inhibitor of anion transport systems[2], but not by avermectin. Therefore avermectin appears to distinguish between the subclasses of chloride channels.

1. Yamamoto, D. and Suzuki, N. Proc. R. Soc. Lond. B, 1987 230 93-100.

2. Cabantchik, Z. I. and Rothstein, A. J. Membr. Biol. 1972 10 311-330.

SINGLE CHANNEL STUDIES OF THE INTERACTION OF ARGIOTOXIN$_{636}$ WITH LOCUST MUSCLE GLUTAMATE RECEPTORS.

C. J. Kerry and P. N. R. Usherwood.

Department of Zoology University of Nottingham, Nottingham, England.

The discovery of potent and proven antagonists of transmission at glutamatergic synapses could be important in the development of chemical structures with potential anaesthetic or pesticide action. Venoms from a variety of arthropods have been shown to exhibit both presynaptic and postsynaptic action at such synapses with recent attention being focused on venoms of orb-web spiders (in particular *Nephila, Argiope* and *Araneus spp.* [1,2,5]) from which low molecular weight (<1 kdaltons) toxins have been purified. We report here on a toxin (argiotoxin$_{636}$) of 636 daltons which has been isolated from the venoms of *Argiope spp.* and *Araneus gemma.* The interactions of this toxin with the ionic channel gated by the quisqualate-sensitive D-receptor found on the extrajunctional membrane of locust muscle are described.

Metathoracic extensor tibiae muscles of adult *Schistocerca gregaria* were pretreated with a solution of 10^{-6}M concanavalin A to block receptor desensitisation. Recordings of the activity of single glutamate D-receptor channels were obtained using the megaohm seal patch clamp technique and a conventional two-electrode voltage-clamp. Dilutions of argiotoxin$_{636}$ (10^{-14}M to 10^{-9}M) and of L-glutamate (10^{-5}M to 10^{-3}M) were prepared in standard locust saline and applied via either the patch pipette or the muscle bath. Data were recorded on tape and subsequently analysed using a PDP 11/34 microcomputer and a Masscomp MC5500. Single channel kinetics were initially characterised in terms of qualitative and quantitative changes in overall parameters (channel open probability (P_o), channel event frequency (f), mean channel open time (m_o) and mean channel closed time (m_c)) and subsequently in terms of dwell time probability density functions (pdf's) , open time and closed time autocorrelation functions (acf's) and adjacent (i.e. open-closed) dwell time correlations (adt's) (see [3,4]).

Argiotoxin$_{636}$ reduced the activity of the glutamate receptor-gated channel (GluR). Although this change was a continuous process three types (TYPE I to III) of channel behaviour were clearly distinguished (see figure). Compared to CONTROL behaviour [3], TYPE I behaviour was characterised by reductions in P_o and f, an increase in m_c and either no change in m_o or, infrequently, an increase in this parameter; TYPE II behaviour was characterised by further reductions in P_o and f, an increase in m_c and a reduction in m_o; TYPE III behaviour was characterised by an apparent absence of channel openings. The toxin-induced channel transitions always progressed in the direction of TYPE III behaviour i.e.

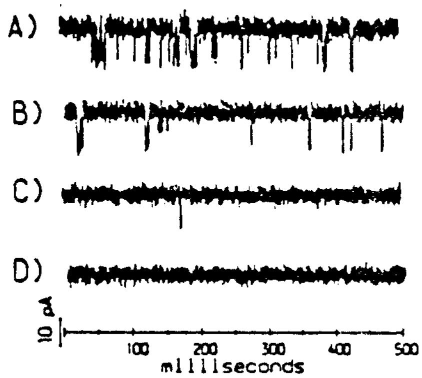
CONTROL → TYPE I → TYPE II → TYPE III.

The time taken for these transitions decreased with increasing concentration of argiotoxin$_{636}$, whether patch pipette or bath applied, i.e. TYPE I behaviour changed to TYPE II behaviour after ~115 s at 10^{-13}M, ~75 s at 10^{-12}M and ~55 s at 10^{-11}M (N≥4).

Quantitative analysis of lengthy recordings from single membrane sites revealed that channel openings in the presence of argiotoxin$_{636}$ were more clustered than normal. In one recording made in the

presence of 10^{-4}M glutamate with 10^{-10}M argiotoxin$_{636}$ in the patch pipette these clusterings were particularly apparent and in this recording m_o was double that for CONTROL behaviour.

Subsequent analysis of CONTROL, TYPE I and TYPE II channel behaviour in terms of their underlying pdf's showed the transitions between the different types of behaviour to be associated with first an increase in the duration and contribution of longer closed times and second a reduction in the number of



Kinetic Parameters of Channel Behaviour Types					
	Type	$f(s^{-1})$	P_o	m_o *(ms)*	m_c *(ms)*
A)	CONTROL	51.9	0.057	1.11	18.18
B)	TYPE I	29.5	0.035	1.20	32.40
C)	TYPE II	6.4	0.0035	0.55	155.4
D)	TYPE III	-	-	-	-

open states. Acf analysis and adt analysis revealed a weakening and eventual loss of the positive correlations between successive open times and negative correlations between adjacent closed times whereas positive correlations between successive closed times remained. In the absence of toxin the glutamate dose-response curve showed an increase in P_o and m_o and a decrease in m_c as the concentration of glutamate was raised from 10^{-5}M to 10^{-3}M [4]. Recordings made under similar conditions but in the presence of argiotoxin$_{636}$ (10^{-11}M or 10^{-10}M) failed to show any significant dose-dependent change in these parameters.

The changes in single channel kinetics indicate that argiotoxin$_{636}$ blocks the cation-selective quisqualate-sensitive GluR in insect muscle at the level of the open channel thus supporting previous macrosystem studies on locust [1,6] and blowfly [5]. The results do not, however, exclude the possibility that argiotoxin$_{636}$ is also either a closed channel blocker and/or a competitive antagonist. The presence in a few recordings during the initial stages of antagonism of increased clustering of channel openings with an increase in m_o raises the possibility that the toxin also interacts allosterically with the glutamate binding site on the glutamate receptor.

The results of the study reported here encourage the view that argiotoxin$_{636}$ will be a useful tool in neurophysiological research and stimulate studies on the other family of low molecular weight argiotoxins in which the 2,4-dihydroxyphenyl chromophore is replaced by an indole chromophore.

(1) Bateman, A., Boden, P., Dell, A., Duce, I.R., Quicke, D.L.J. & Usherwood, P.N.R. Brain Res. 1985, 339 237-244.
(2) Kawai, N., Miwa, A., Saito, M., Pan-Hou, H.S. & Yoshioka, M. J. Physiol., Paris 1984, 79 228-231.
(3) Kerry, C.J., Kits, K.S., Ramsey, R.L., Sansom, M.S.P. & Usherwood, P.N.R. Biophys. J. 1987, 51 137-144.
(4) Kerry, C.J., Ramsey, R.L., Sansom, M.S.P. & Usherwood, P.N.R. Biophys. J. 1987, (In press).
(5) Magazanik, L.G., Antonov, S.M., Fedorova, I.M., Volkova, T.M. & Grishin, E.V. Biological membranes 1986, 3(12) 1204-1219.
(6) Usherwood, P.N.R., Duce, I.R. & Boden, P. J. Physiol., Paris 1984, 79 241-245.

Patch-clamp recordings of transmitter-activated ion channels in mammalian sympathetic neurones.

by Alistair Mathie; M.R.C. Receptor Mechanisms Research Group;
Department of Pharmacology; University College London; Gower St.;
London WC1E 6BT

In mammalian sympathetic ganglia, the fast chemical transmitter released from preganglionic fibres to excite postganglionic neurones is acetylcholine (ACh). ACh induces a membrane current change by binding to neuronal nicotinic ACh-receptors. This causes a conformational change in the protein which allows the flow of small cations through an ionic channel that is thought to be an integral part of that protein.

Mammalian neuronal nicotinic receptors are known to differ from the more widely studied muscle nicotinic receptors in terms of their pharmacology. Recently, the structural and functional properties of the neuronal receptors have begun to be characterised and these, too, show many differences from muscle. In dissociated rat sympathetic neurones, the channels opened by ACh have a unitary conductance (γ) of 35pS. They respond to a single receptor activation with a burst of openings. The distribution of burst lengths can be fitted by the sum of two exponential components with time constants τ_{b1} = 0.4ms and τ_{b2} = 11.9ms (at -100mV and room temperature). The longer burst component carries about 97% of the charge and probably, therefore, has most relevance for synaptic transmission. Mammalian chromaffin cells (which share the same embryonic origins as

sympathetic neurones) have neuronal nicotinic receptors with very similar properties:- a single channel conductance (γ) of 39pS and a burst distribution fitted by two exponentials with time constants τ_{b1} = 0.7ms a τ_{b2} = 9.5ms (at -100mV and room temperature).

Having established the basic kinetic properties of these receptors it is possible to study chemicals which block the ACh-current in neurones with a view to understanding their mechanism of action in more detail. Two chemicals which reduce the ACh-current in both the cell types described above are (a) clonidine, an α-adrenoceptor agonist used therapeutically in the control of hypertension, which reduces the ACh-current as an additional unrelated effect and (b) K-bungarotoxin (kappatoxin) a snake toxin which selectively blocks the neuronal ACh-current and is proving a useful tool in locating nicotinic receptors in the brain. These two chemicals appear to have quite different mechanisms of action. Clonidine has no effect on the unitary conductance of single ACh-channels, but markedly reduces the burst length of the channels in response to receptor activation suggesting that it acts, either, by directly blocking the channel or by binding to a site in or close to the channel thereby altering its gating. Kappatoxin also has no effect on the unitary conductance of the ACh-channels but neither does it appear to alter the gating of the channel. This suggests that kappatoxin may act by directly binding to the receptive sites for ACh on the protein. This would reduce the number of receptors available to ACh and hence reduce the ACh-current without affecting the properties of the remaining ACh-receptors free for activation.

Glutamate activated membrane channels in crustaceans

J.Dudel

Physiologisches Institut der Technischen Universität München
Biedersteiner Str. 29, 8000 München 40, FRG

Two classes of membrane channels activated by glutamate are known in crustaceans: "excitatory" channels which pass small cations, and "inhibitory" channels permeable to chloride ions. Both have been studied in cell-attached or excised, outside-out and inside-out membrane patches of muscle membrane of crayfish (3-5). Glutamate and related substances were applied to the outside of the membrane either continuously, or in well defined pulses employing the liquid-filament switch (6).

The excitatory channels are activated by quisqualate and glutamate, and not by kainate and NMDA. They are permeable to Na^+, K^+, Li^+, Ca^{2+}, Ba^{2+} and Mg^{2+} (in decreasing order of permeability) (7). The single channel conductance is about 100 pS (4,7). The single channel openings have an average duration of about 0.2 ms. They are grouped in bursts, the duration of which increases from 0.3 to 1.3 ms in dependence on the glutamate concentration (0.1 to 20 mM) (1,4). At a constant glutamate concentration, the rate and the duration of bursts declines with decreasing extracellular Ca^{2+} concentration; at less than 2 mM Ca^{2+} practically no channel openings are observed (8). When glutamate is applied in pulses, maximum activation of (up to about 20 channels/patch) can be reached within less then 1 ms.Activation is followed by rapid desensitization. In one type of channels, the average current declines with a time constant of about 5 ms to a low level of continuous activation (6). In a second type of channels, the average current declines with about the same rate, but desensitization is complete, with no channel openings in the presence of glutamate after one initial burst (2).

An "inhibitory" channel permeable to chloride ions is activated by quisqualate, glutamate, GABA and β-guanidino-propionic acid (in decreasing order of potency). The channel conductance has 3 substates with a basic state γ_i = 22 pS and two higher states of

$2\ \gamma_i$ and $3\ \gamma_i$ (in symmetrical high chloride). While quisqualate and glutamate preferentially activate the first substate, a high proportion of openings to the second substate is elicited by GABA. Picrotoxin blocks activation by GABA, and less well that by glutamate (5). Low Ca^{2+} concentration increases channel activation, especially by GABA. The substates have generally two components in the open time distributions. The openings are grouped in bursts for which also two components of on average 3 and 35 ms duration are observed (5). Pulses of glutamate or of GABA activate the chloride channels relatively slowly. Peak activation of up to several hundered channels per patch is reached after more than 10 ms. Then the average currents decline with a time constant of about 300 ms, but about a third of maximum activation remains in continued presence of the activator.

(1) Dudel J., Franke Ch. (1987) Pflügers Arch. 408:307-314

(2) Dudel J., Franke Ch., Hatt H., P.N.R. Usherwood (1988) Pflügers Arch., in press

(3) Franke Ch., Dudel J. (1987) Pflügers Arch. 408:300-306

(4) Hatt H., Dudel J. (1986a) J. Comp. Physiol. 159:579-589

(5) Franke Ch., Hatt H., Dudel J. (1986b) J. Comp. Physiol. A 159:591-609

(6) Franke Ch., Hatt H., Dudel J. (1987) Neurosci Lett. 77 199-204

(7) Hatt H., Franke Ch., Dudel J. (1988) Pflügers Arch. 411:8-16

(8) Hatt H, Franke Ch., Dudel J. (1988b) Pflügers Arch. 411:17-26

Ion Channels in Artificial Lipid Bilayers.

M.S.P. Sansom

Department of Zoology, Nottingham University, University Park, Nottingham, NG7 2RD.

The reconstitution of ion channels into planar lipid bilayers is an important tool for probing the relationship between channel function and molecular structure. The method may be used to:-

a) transplant a channel from its native membrane into a novel lipid environment so as to study channel-membrane interactions;

b) demonstrate that a purified receptor protein will form functional ion channels; and

c) study the properties of simple models of ion channels.

We are interested in all three of these applications.

Various experimental configurations are available for ion channel reconstitution. We have used formation of planar bilayers at the tips of patch electrodes, as this gives improved temporal resolution and higher signal-to-noise ratios. The bilayer is formed by moving a patch electrode out of and then back into an electrolyte solution which has a lipid monolayer formed upon its surface[1]. Using this method we have investigated:-

a) ion channels formed by simple peptide molecules; and

b) putative receptor-channels from locust CNS and muscle membranes.

To date, the majority of studies on model channels have concentrated on the antibiotic gramicidin. Whilst providing us with structural insights into ion channel properties, the β-helix formed by the alternating D,L amino acid sequence of gramicidin cannot be adopted by channel proteins, and therefore investigations of peptides containing only L-amino acids are of importance. As there has been considerable interest in the interpretation of ion channel amino acid sequences in terms of trans membrane α-helices, we are keen to study model channels formed by α-helical peptides. Such peptides are generally amphipathic and haemolytic, and are thought to form α-helical clusters in the membrane, with the helix axes approximately perpendicular to the plane of the bilayer.

We have studied two such peptides, both of which form highly amphipathic α-helices:- the δ-toxin of

Staphylococcus aureus , and mastoparan from the venom of the wasp *Vespa lewisii* . Both form ion channels in bilayers of diphytanoyl phosphatidylcholine at peptide concentrations of ca. 1 μM. δ-Toxin channels exhibit two conductance classes - "small" channels of conductance 70 - 100 pS, and "large" channels of conductance ca. 450 pS. The latter may be reasonably approximated to by diffusion limited pores formed by hexameric clusters of α-helices. Current-voltage relationships of membranes exposed to relatively high concentrations of δ-toxin were symmetrical about 0 mV, and revealed both voltage-dependent and voltage-independent components to channel gating. The conductance at 0 mV was proportional to ca. the 7th power of the toxin concentration. The channels were weakly cation selective, the "small" channels more so than the "large". These results have been interpreted in terms of the hexameric model of the δ-toxin channel first proposed by Freer and Birkbeck[2].

Mastoparan is of interest in that it is only 14 residues long, and so would be predicted to form helices rather too short, at 2.1 nm, to span the lipid bilayer. However, it has been shown to form ion channels. Current-voltage relationships were makedly asymmetric, revealing that channels only form when the peptide-containing compartment is held at a positive potential. Again, these results have been interpreted in terms of the known structure of mastoparan.

More recently, we have investigated the possibility of reconstitution of ion channels from locust CNS and muscle membrane fractions. Encouraging preliminary results will be presented. The aim of this work is to reconstitute the locust muscle glutamate receptor, so as to enable detailed investigations of the effect of the novel membrane environment on ion channel biophysics.

References.

1) Coronado, R. and Latorre, R. (1983) Biophys. J. 43, 231-236. Phospholipid bilayers made from monolayers on patch-clamp pipettes.

2) Freer, J.H. and Birkbeck, T.H. (1982) J. Theor. Biol. 94, 535-540. Possible conformation of delta-lysin, a membrane damaging peptide of *Staphylococcus aureus* .

Molecular Recognition, Structural Dissimilarity and Future Developments in QSAR

Dr P.M.Dean, Department of Pharmacology, University of Cambridge, Cambridge.

In the past the incorporation of dissimilar molecular structures into QSAR has been omitted. There are two principal reasons for this neglect: firstly, quantitative methods for matching dissimilar molecules have been slow to emerge; secondly, few workers have attempted to understand the problems of pattern sensing. This paper tackles both deficiencies.

For a drug molecule, or pesticide, to show biological activity it usually has to bind to an active site on an enzyme or receptor molecule. If two molecules bind to a shared set of site points we may infer that they possess a complementary set of ligand points with similar functional chemical properties even though their bonding topologies may be dissimilar. Where the active face of one molecule is known, or can be inferred from systematic chemical modifications linked to QSAR studies, then a second dissimilar molecule can be rotated until a maximal pattern match between the two binding faces is obtained. This pattern match can be found for any given surface motif by minimization in rotational 3-space. A more complicated situation arises where the binding motif is unknown. In this case pattern sensing on the surface of both molecules has to be studied by blind searching to identify common features on each molecule within a specified search window. Optimized pattern matching and pattern sensing methods have been developed by combining 3- or 6-dimensional minimization techniques with hierarchical cluster analysis.

STRUCTURE-ACTIVITY STUDIES ON MAMMALIAN GLUTAMATE RECEPTORS.

Jeffrey C. Watkins.

Department of Pharmacology, The Medical School, Bristol, England BS8 1TD.

There are at least four types of excitatory amino acid (EAA) receptors in the mammalian central nervous system, those activated by the preferential agonists N-methyl-D-aspartate (NMDA), quisqualate (Q), kainate (K) and L-2-amino-4-phosphonobutanoate (L-AP4). L-Glutamate is a mixed agonist capable of activating each type of these receptors and is probably the transmitter acting physiologically at all four types.

NMDA RECEPTORS

These are by far the best characterized receptors. Structure-activity relations for NMDA receptor agonists can be summarized as follows: (i) L-Glutamate has the highest affinity of all agonists yet tested (ii) Glutamate-length agonists generally have higher activity than aspartate-length agonists (iii) Either D or L forms of agonists with primary amino groups can be accommodated by the receptor, enantiomeric preference varying in different pairs (iv) Substitution in the amino group is usually deleterious; NMDA is an exception, having equal affinity to D-aspartate (which, however, is taken up rapidly and is less effective than NMDA as an agonist in intact tissue) (v) Substitution in the carbon chain is generally also deleterious, but the C-4 position of glutamate is the least vulnerable (vi) The ω-terminal group of agonists is preferably carboxyl, though some ω-sulphinic and ω-sulphonic acids also have high activity; ω-phosphonic acids generally have only weak agonist activity. Competitive antagonists are generally acidic amino acids in which (a) the α-aminomethylcarboxylic acid terminal has the R configuration (i.e. they are D amino acids) (b) the chain separating the two acidic groups is one to three atoms longer than that present in the molecule of glutamate and (c) the ω-acidic terminal is phosphonic (preferably) or carboxylic, with ω-sulphonic acids being much less active. The most potent competitive antagonists yet reported have the α-amino group incorporated in a ring structure, for example, 3-((±)-2-carboxypiperazin-4-yl)propyl-1-phosphonate (CPP) and <u>cis</u>-2-carboxypiperidin -4-yl-methylphosphonate (CGS 19755). A large range of non-competive antagonists has also been characterized, including ketamine, phencyclidine and the very potent MK 801.

Q RECEPTORS

It is usually difficult to differentiate K and Q receptors in the central nervous system. Differentiation was originally made on the basis of the selective depression of quisqualate responses by L-glutamic acid diethyl ester (GDEE). Quisqualate may not be as selective for such GDEE-sensitive sites as the synthetic analogue AMPA (α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid), which, unlike quisqualate, does not inhibit binding of ^{3}H-kainate.

Potent and selective antagonists for Q receptors have proved difficult to develop and, until recently, apart from GDEE (a notoriously weak and unreliable antagonist, probably acting non-competitively), only non-selective NMDA/K/Q antagonists were available. In conjunction with specific NMDA antagonists, such broad-spectrum antagonists were useful for classifying receptors involved in synaptic excitation as either NMDA or K/Q types, but not for differentiating K- and Q-type synaptic receptors. These broad spectrum antagonists included kynurenic acid, 1-p-chloro- (and p-bromo)-benzoyl-piperazine-2,3-dicarboxylic acids, γ-D-glutamylglycine (γDGG) and γ-D-glutamylaminomethyl sulphonate (GAMS). However, two new Q receptor antagonists recently described (FG 9065 and FG 9041) are considerably more potent and selective, and appear to constitute a major step forward in this area.

K RECEPTORS

The strict structural requirements for potent inhibition of ^{3}H-kainate binding to rat brain membranes suggest that such binding techniques are the best way to investigate affinities for the K receptor. Domoate (a related substance likewise extracted from a Pacific alga) is the most potent analogue, and the synthetic kainate analogue α-keto kainate also retains high activity. Both these substances are agonists in electrophysiological experiments. Most of the broad spectrum EAA antagonists mentioned under Q receptors (above) appear somewhat more effective as depressants of kainate-induced responses than of quisqualate-induced responses, though the degree of differentiation in ionophoretic experiments in vivo is not sufficiently high in any case for definitive characterization of synaptic responses. Recent experiments have indicated that 3-hydroxy-6,7-dichloro-quinoxaline-2-carboxylic acid may be the most potent and selective kainate receptor antagonist yet reported. However, the two quinoxaline derivatives FG 9065 and FG 9041 also have high kainate antagonist activity and further selectivity data on these three compounds is awaited with interest.

L-AP4 RECEPTORS

The classification of these receptors arose from the observation that L-AP4 depressed synaptic responses in the hippocampus and spinal cord, but did not depress responses to NMDA, kainate or quisqualate. These receptors may be autoreceptors, located presynaptically, and acting to reduce the release of excitatory transmitters. In the retina, L-AP4 mimics L-glutamate in hyperpolarizing ON bipolar cells. The pharmacology of L-AP4 effects in the hippocampus has been studies in some detail, but potent agonists or antagonists have yet to be reported.

The Use of Multivariate Analysis in Toxicological Studies

David W Salt[1] and Martyn G Ford[2]

1 Department of Mathematics and Statistics
2 School of Biological Sciences
Portsmouth Polytechnic
Portsmouth
Hampshire

When neurotoxicants are applied to excitable tissues, a range of responses is observed. The variables describing these responses may include the frequency, height and shape of action potentials; the pattern of depolarization and hyperpolarization observed at short and long term responses; and the concentrations of drug or pesticide necessary to elicit fixed responses under standard conditions. Assays designed to evaluate these phenomena therefore give rise to a multivariate data set which we shall term the response set. It is usual for neurotoxicological assays to be conducted on only one of these variables, selected *a priori* as an indicator of toxicity. However, the choice of an appropriate response variable is often arbitary, based on no prior knowledge of its usefulness as an indicator of the activity of the compound in the whole organism.

The neurotoxicological responses produced by a series of related compounds will depend on the molecular properties of the applied materials. The relationship between chemical structure and biological activity is important for the rational design of novel drugs and pesticides. The molecular properties of a series of compounds can be summarised in terms of a number of descriptor variables which may either be observed, or predicted by empirical or theoretical calculations; in general the greater the number of variables, the more complete the description of the molecular properties of the series. Molecular properties will therefore give rise to a second multivariate data set, the descriptor set.

Any investigations of the properties of the two data sets and their associations will be difficult, but necessary, if a full understanding of the processes underlying toxicity and the relationship between these processes and molecular structure is to be gained. One approach involves the use of multivariate statistical procedures which attempt to summarise the tendencies and associations within and between data sets in terms of vectors lying between coordinates representing the variables of interest. This paper will describe a number of these statistical procedures in an attempt to illustrate their utility in the study of structure-activity relationships. Examples based on the application of pyrethroid insecticides to intact insects and insect excitable tissues will be presented and discussed. It is hoped that this account will encourage neurotoxicologists and chemists to use multivariate statistical techniques as an aid to the prediction of novel structures for drugs and insecticides, thus gaining a better understanding of their mode of action.

A MULTIVARIATE QSAR STUDY OF PYRETHROID NEUROTOXICITY BASED UPON MOLECULAR PARAMETERS DERIVED BY COMPUTATIONAL CHEMISTRY

David J. Livingstone

Wellcome Research Laboratories, Beckenham, Kent, BR3 3BS, England,

Martyn G. Ford & David S. Buckley

School of Biological Sciences, Portsmouth Polytechnic, Portsmouth, Hants., PO1 2DY, England.

Chemists and neurobiologists interested in the relationship between biological activity and chemical structure are involved in a search for correlations between biological data and physicochemical descriptors, with the aim of developing more potent analogues through an understanding of their mode of action. Using the "traditional" techniques of QSAR, the number of molecular descriptors available to an investigator is limited; the increasing use of "Computer Chemistry" in QSAR studies has made it possible to calculate large numbers of descriptor variables for such sets of compounds. However, the ability to describe chemical structures in this detail may lead to problems in the statistical analysis of the data making it necessary to use multivariate methods. Similarly, biological tests may generate a number of "biological" responses, particularly for in vitro neurotoxicological tests[1]. Thus the problem is multivariate with respect to both the biological and physicochemical data.

In the example reported in this paper, a series of substituted benzyl cyclopropane-1-carboxylates has been studied in which simple changes in the ring substituents produce profound changes in neurotoxicological and insecticidal potency of the compounds. These synthetic pyrethroids are well suited to the prediction of properties by computational chemistry; 70 parameters were calculated for a set of 21 compounds which were "built" on the basis of the crystal structure of CYPERMETHRIN[2]. The biological data

set corresponding to this physicochemical data consisted of 10 response measurements.

The methodology used to reduce these data matrices to manageable proportions will be described and the following conclusions discussed.

(1) The molar threshold concentration (MTC) necessary just to elicit a response in perfused isolated haltere nerves of the housefly (musca domestica) was found to correlate with the atom charge of a particular aromatic ring carbon.

(2) Neurotoxicity was found to be composed of two factors, the first linking both killing and knockdown activity while the second was mainly associated with knockdown. This second factor, therefore, provides a discrimination between knockdown and killing action for this set of pyrethroids.

(3) The first factor was associated with those neurotoxicological responses such as nerve block which are diagnostic of type II action[3]. The second factor was associated with the bursting behaviour of pyrethroid treated nerves which is characteristic of type I action[3].

1. Buckley, D.S., Ford, M.G., Leake, L.D., Salt, D.W., Burt, P.E., Moss, M.D.V., Brealey, C.J. & Livingstone, D.J. in "QSAR in Drug Design and Toxicology" ed. Hadzi, D. & Jerman-Blazic, B., 1987, Elsevier, 336-339.

2. Owen, J.D., J.Chem.Soc. Perkin Trans I, 1975, 1865-1868.

3. Gammon, D.W., Brown, M.A. & Casida, J.E., Pestic.Biochem.Physiol. 15, 1981, 181-191.

QUALITATIVE AND QUANTITATIVE MODELLING OF DRUGS AND PESTICIDES

Flemming S. Jørgensen, Jette R. Byberg and Povl Krogsgaard-Larsen
Department of Chemistry BC, Royal Danish School of Pharmacy,
Universitetsparken 2, DK-2100 Copenhagen, Denmark

James P. Snyder
Drug Design Section, G.D. Searle Research and Development,
4901 Searle Parkway, Skokie, Illinois 60077, U.S.A.

Structure-activity relationships are extremely important for gaining a better understanding of the mechanisms associated with the activity of drugs and pesticides. Molecular modelling and computer graphics techniques have in the last two decades made it possible to study the three-dimensional aspects of drug receptor interactions in a very efficient way. Simultaneously, the application of quantitative structure-activity relationships based on the Hansch approach has matured considerably. Today the combination of these two approaches seems to lead to a very powerful tool for the design and development of new biologically active compounds.

To illustrate some of the possibilities as well as some of the problems associated with these methods three different examples will be presented:

In 1983 Schulman and coworkers published a three-dimensional model for recognition of **muscarinic agonists.**[1,2] The model was based on conformational analysis of nine more or less rigid compounds being potent muscarinic agonists.

After publication of the model several of the assumptions behind the model have been questioned.[3,4,5] The basis of the model will be outlined together with a short discussion of the criticism of the different assumptions.

Finally, some suggestions for developing an improved model for muscarinic agonists will be presented.

For a series of structurally different **pyrethroid esters** being potent insecticides the strategy behind a determination of the pyrethroid pharmacophore will be presented.

Problems to be addressed will include:

- .. selection criteria for the compounds used for the pharmacophore mapping,
- .. superposition of the essential features of the compounds in order to achieve maximum molecular similarity, and
- .. evalaluation of different superpositions/pharmacophores.

The pharmacophore mapping led to a three-dimensional model for the acid and alcohol moieties, respectively.[6] Some of the problems associated with combining these pharmacophores and establishing a quantitative model for pyrethroid activity will briefly be discussed.

Based on a series of conformationally restricted glutamic acid derivatives a three-dimensional model for the recognition of **QUIS/AMPA** agonists has been deduced.

The model makes it possible to classify some of the surroundings as either "allowed" or "disallowed". One consequence of the model, the possibility for predicting the stereoselectivity of QUIS/AMPA agonists will be discussed.

Attempts to develop a quantitative model by placing counter-molecules, resembling an artificial receptor site, around the model have been made. Some of the problems, possibilities and limitations of such a model will be presented.

1. Schulman, J.M., Sabio, M.L. and Disch, R.L. **J. Med. Chem.** (1983) 26 817-823.

2. Tollenaere, J.P. **Trends Pharmacol. Sci.** (1984) 5 85-86.

3. Snyder, J.P. **Trends Pharmacol. Sci.** (1985) 6 464-466.

4. Weinstein, H. **Trends Pharmacol. Sci.** (1986) 7 262-263.

5. Gualteri, F. **Drug Design and Delivery** (1987) in press.

6. Byberg, J.R., Jørgensen, F.S. and Klemmensen, P.D. **J. Computer-Aided Molecular Design** (1987) in press.

The dynamic basis of selective toxicity

Martyn Glenn Ford

School of Biological Sciences, Portsmouth Polytechnic, Portsmouth, Hampshire, PO1 2DY.

Quite small modifications to the molecular properties of drugs and pesticides can result in substantial changes in biological activity. Different compounds of similar chemical class acting at the same site of action can show relative toxicities (measured as 1/LD50) in excess of 100,000; the susceptibility of different species to the same toxicant can vary by a similar factor. Such selectivity can prove beneficial. The control of target pests at commercial doses too low to threaten non-target organisms is a useful agrochemical property, and a large therapeutic ratio is a necessary property for the safe use of clinical pharmaceuticals. Selective toxicity can sometimes lead to serious problems in the use of drugs and pesticides, however. The development of strains of organisms resistant to biologically active compounds, for example, can result in their premature withdrawal from commercial use.

A number of processes may be associated with the establishment of selectivity. These may be broadly classified as pharmacokinetic processes, which act to modify the exposure of the site of action to a drug or pesticide, and pharmacodynamic processes ,which determine the strength and mode of binding of the ligand to the receptor and, as a result, may perturb second messenger signalling systems. This paper will review the influence of pharmacokinetics and pharmacodynamics on the selective toxicity of insecticides.

The toxicity of a compound may vary in a dynamic manner with time after application. The influence of elapsed time on the absolute toxicity of a poison can be attributed to changes in the definite integral which defines internal exposure to the toxicant. Factors such as rates and extent of penetration, binding, detoxification and elimination all modify exposure to result in changes in relative potency with increasing time after treatment. Examples based on investigation of the mode of action of pyrethroid insecticides applied to various insect species suggest that penetration and non-specific binding may be important during the early stages of poisoning. At longer elapsed times when death occurs, elimination of insecticide from the insect body may dominate pharmacokinetics and thus be more crucial in determining relative toxicity.

Studies of the action of synthetic pyrethroids on lepidopterous larvae suggest that the gut plays a major role in elimination of penetrated insecticide, largely through regurgitation of (and possibly chemical degradation by) the gut contents. Loss of gut contents is associated with acute dehydration of larvae, but there is little evidence to substantiate the view that death occurs as a result pyrethroid induced water loss. Steady states levels of pyrethroids are achieved within a few hours of topical application. The ability of this caterpillar to degrade pyrethroids seems limited by the low rates of in vivo detoxification observed in the various insect tissues. The more rapid rates of detoxification reported during in vitro degradation by tissue extracts may be misleading, since barriers restricting the availability of pyrethroid to detoxifying enzymes may be destroyed during the extraction process.

The relative neurotoxic potency of different but related insecticides is invariant of insect species, although absolute neurotoxicity can vary markedly between species. This suggests that pharmacodynamics may profoundly influence the whole animal toxicity to different species of a particular compound in a class of insecticides, but is less likely to contribute to relative changes in the potencies of a series of compounds tested against the same species.

PHARMACOKINETICS OF INSECTICIDES IN INSECTS.

Clive J. Brealey.

Wellcome Research Laboratories, Ravens Lane, Berkhamsted, Herts, England, HP4 2DY.

Pharmacokinetics can be considered to include all the processes which occur between deposition of an insectide on the surface or in the gut of an insect and its arrival at the target site macromolecule. In the pesticide industry valid inferences about the penetration and metabolism of an insecticide are often made on the basis of experiments using different dosing routes, formulations and synergists. However there seems to be no substitute for quantitative measurements on the rates of penetration, routes of metabolism and insecticide concentrations in specific tissues. Examples of the different approaches to the use of pharmacokinetics in insects are discussed.

Simple comparisons between species or strain for external and internal amounts of parent insectide and key metabolites can largely explain resistance phenotypes. The resistance of the yellow fever mosquito to DDT and permethrin is readily described by measurements of penetration and metabolism : not only is DDT converted to DDE more extensively in two resistant strains but target site insensitivity (kdr)is also present and this latter mechanism exclusively accounts for cross-resistance to permethrin[1].

A more general quantitative consideration of penetration using computer models as previously described[2] is a useful tool when data are available on a larger number of structurally-related analogues. However these models do not account for complications of the penetration process caused by toxic action of a compound. In the locust insecticide poisoning causes regurgitation of fluid[3]. A similar loss of fluid from the mouthparts of the housefly and Spodoptera littoralis larva appears to cause a redistribution of insecticide from inside to outside of the insect with a corresponding discontinuity in the penetration curve.

Definitive assessments of metabolism require in vivo studies with radiolabelled compounds . However the combined use of in vitro model systems for metabolism and in vivo studies can provide rapid answers to metabolism questions. The pharmacokinetics and metabolism of two insecticidal lipid amides (figure 1, I and II) have been studied in the housefly in vivo and rat liver microsomes in vitro. Differences in internal concentrations of the compounds are related in part to the enhanced opportunities for epoxidation with the introduction of additional unsaturation into the molecule[4].

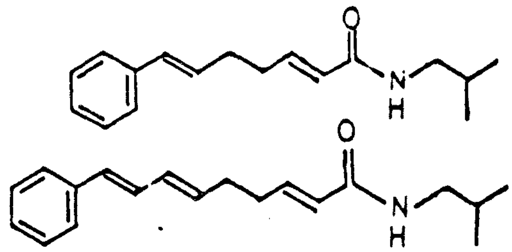
Metabolism in insects is a dynamic process where the insecticide-metabolising enzymes are susceptible to rapid induction . In particular multiple forms of cytochrome P-450 are selectively induced as in mammalian systems. An example is found in the housefly where clofibrate causes a specific change in the ratio of lauric acid hydroxylation at the 11- and 12-positions without a large change in the total cytochrome P-450 content. Phenobarbital causes a specific increase in benzphetamine N-demethylation[5].

Finally, the concentration of an insecticide toxicant can be measured in a target tissue in vivo at doses causing toxicity. These estimates can be compared with estimates of the potency of the toxicant in a comparable in vitro assay system. For example the concentration of permethrin in the head ganglia of houseflies at a toxic dose can be compared with estimates of concentrations disruptive to the nervous system in vitro[6].

Figure 1. Insecticidal lipid amides[7].

I

II



References

1. Brealey, C.J., Crampton, P.L., Chadwick, P.R., Rickett, F.E. Pestic. Sci. 1984, 15 121-132.

2. Ford, M.G., Greenwood, R., Thomas, P.J. Pestic. Sci. 1981, 12 175-198.

3. Samaranayaka, M. Pestic. Biochem. Physiol. 1977, 7 510-516.

4. Brealey, C.J. Biochem. Soc. Trans. 1987 (in press).

5. Clarke, S.E., Brealey, C.J., Gibson, G.G. Biochem. Soc. Trans. 1987 (in press).

6. Buckley, S., Brealey C.J., Ford, M.G. and Leake, L.D. IUPAC Sixth Int. Congr. Pestic. Chem., (Greenhalgh, R. and Roberts, T.R., Eds.), Blackwell, Oxford, (1987), 205.

7. Black, M.H., Blade, R.J., Moss, M.D.V.M., Nicholson, R.A. 1986, IUPAC Sixth Int. Congr. Pestic. Chem., (Greenhalgh R, and Roberts, T.R., Eds.), Blackwell, Oxford, (1987), Abstract 1B-21.

NEUROPHYSIOLOGICAL ASSAYS FOR THE CHARACTERIZATION AND MONITORING OF PYRETHROID RESISTANCE

Jeffrey R. Bloomquist

Department of Entomology, New York State Agricultural Experiment Station, Cornell University, Geneva, NY 14456 USA

Knockdown resistance to pyrethroids remains a major problem with respect to their continued use as agricultural insecticides. This type of resistance was originally described in the housefly, but has been proposed as a resistance mechanism in other species as well. The presence of knockdown resistance is often inferred from toxicity studies with synergists, but must be confirmed using electrophysiological methods. Production of abnormal nerve function in preparations from resistant insects requires longer periods of insecticide exposure or higher insecticide concentrations than in equivalent assays from susceptible strains. Monitoring for pyrethroid resistance with neurophysiological assays is difficult, for although they can detect resistance in individual insects, they are labor intensive and cannot be used to process the large number of samples needed to detect resistant alleles present at a low frequency. Moreover, although physiological studies can demonstrate reduced sensitivity of the nervous system, they cannot by themselves define the underlying resistance mechanism.

The most intensively studied example of knockdown resistance to pyrethroids is the *kdr* factor of the housefly, but this factor is not necessarily equivalent to *kdr*-like traits observed in other species. Our recent studies on a temperature-sensitive paralytic mutant of *Drosophila melanogaster* provide evidence for an unusual *kdr*-like resistance mechanism in this insect that confers broad cross resistance to pyrethroids and DDT. Physiological assays confirmed that the resistance is expressed at the level of the nerve by recording spontaneous and evoked activity in the fibrillar flight muscles of poisoned flies.

NEUROPHARMACOLOGY AND MOLECULAR GENETICS OF NERVE INSENSITIVITY RESISTANCE TO PYRETHROIDS

David M. Soderlund

Department of Entomology, New York State Agricultural Experiment Station, Cornell University, Geneva, NY 14456 USA

Resistance to pyrethroids conferred by reduced sensitivity of the nervous system has been detected in several insect species and has been documented in considerable detail in the housefly. In this species, the *kdr* and *super-kdr* traits represent allelic variants at a single locus that confer broad cross resistance to DDT, DDT analogs, and pyrethroids. Three mechanisms have been proposed to account for reduced neuronal sensitivity: reduced number or density of the insecticide target site; alterations in the lipid composition of neuronal membranes; and alterations in the insecticide-binding domain of the target macromolecule.

Because of the central role of the voltage-sensitive sodium channel in the actions of pyrethroids on nerves, mechanisms of reuced neuronal sensitivity are likely to involve changes in the number, environment, or properties of this site. The density of sodium channels in neuronal membrane preparations can be estimated by the specific binding capacity of these preparations for [^{3}H]saxitoxin. Comparison of the number and properties of the binding sites for this ligand in head membrane preparations from susceptible and *kdr* houseflies revealed no differences between strains. These findings tend to rule out reduced target site number as a mechanism underlying the *kdr* trait in the housefly, but this mechanism may contribute to reduced neuronal sensitivity in other species.

To distinguish between between the remaining two proposed mechanisms of reduced neuronal sensitivity, it is necessary to separate the effects of membrane environment from those involving structural changes in one or more binding domains of the sodium channel.

Mammalian sodium channels can be purified and reconstituted into artificial membranes, but these methods have not been successful to date with insect sodium channels. However, two aproaches based on the molecular biology of vertebrate sodium channels offer alternatives to purification and reconstitution. First, injection of mRNA into oocytes of the frog *Xenopus laevis* results in the synthesis and functional incorporation into the oocyte membrane of a variety of neurotransmitter receptors and ion channels. The successful expression and pharmacological characterization of insect sodium channels in the oocyte membrane would enable the comparison of the properties of channels from susceptible and *kdr* insects in the absence of strain-specific differences in neuronal membrane properties. Second, a gene has been cloned and sequenced from *Drosophila melanogaster* that is very similar in structure and organization to the sodium channel genes previously described from electric eel and rat brain. Although the functional identity of the *Drosophila* gene product remains to be demonstrated, probes derived from this gene may provide points of entry for the cloning and structural characterization of sodium channel genes from susceptible and *kdr* insects. Our current research efforts are directed at exploring the utility of both the *Xenopus* expression assay and probes derived from the putative *Drosophila* sodium channel gene to characterize sodium channels in the housefly and determine the molecular basis of the *kdr* phenotype.

\sattelle\ntox

ION CHANNELS OF INSECTS SUSCEPTIBLE AND RESISTANT TO INSECTICIDES

D.B. Sattelle,[1] C.A. Leech,[1] S.C.R. Lummis,[1] B.J. Harrison,[1] J.A. David,[1] G.D. Moores,[2] A.W. Farnham,[2] A.L. Devonshire[2]

AFRC Institute of Arable Crops Research: [1]Unit of Insect Neurophysiology and Pharmacology, Department of Zoology, University of Cambridge CB2 3EJ, U.K. and [2]Department of Insecticides and Fungicides, Rothamsted Experimental Station, Harpenden, Herts, AL5 2JQ, U.K.

Although the sodium channel is generally considered to be the critical target for pyrethroid insecticides[1], GABA receptors and calcium channels have also been advanced as potential target sites for the pyrethroid insecticides. All three channels can be found in the membrane of a single identifiable cockroach (Periplaneta americana) motor neurone, and their relative sensitivity to the pyrethroid insecticide deltamethrin has been compared.

Resistance to pyrethroid insecticides would threaten their major role in pest control worldwide.[2] To study the molecular basis of pyrethroid resistance, we have examined the properties of sodium channels in susceptible and kdr (knockdown resistant) strains of the housefly Musca domestica. The kdr strains are extensively backcrossed into a susceptible background to minimise genetic differences.

It has been suggested that a change in channel number is the basis for kdr[3]. A multidisciplinary experimental approach has been adopted in order to characterize the density, molecular size and function of sodium channels of Musca domestica. This includes the use of radiolabelled probes specific for different

subsites on the channel molecule, together with radiation inactivation, and microelectrode recording from axons in the connectives linking the brain and thoracic ganglia of adult flies. Successful dissociation of Musca neurones in short-term culture has also been achieved, and these cells are providing the substrate for patch-clamp electrophysiological investigations. Evidence to date indicates that changes in channel density probed by ^{3}H-saxitoxin binding are unlikely to be the major factor in kdr. The problem is suited to molecular biological approaches to the study of sodium channels in susceptible and resistant forms.

References

1. Sattelle, D.B. and Yamamoto, D. (1988) Adv. Insect Physiol. 20, 147-213.
2. Sawicki, R.M. (1985) In Insecticides (ed. D.H. Hutson and R.D. Roberts) pp.143-191. John Wiley, New York.
3. Chang, C.P. and Plapp, F.W. Jr. (1983) Pestic. Biochem. Physiol. 20, 86-91.

SECTION II

****

ABSTRACTS OF CONTRIBUTED PAPERS

A. NATURAL PRODUCTS AND NEUROPEPTIDES

EFFECTS OF PROCTOLIN ON THE FORE AND HIND GUT OF THE LOCUST *SCHISTOCERCA GREGARIA*

S.E. Banner, L. Seale, R.H. Osborne and K.J. Cattell

Department of Science, Bristol Polytechnic, Coldharbour Lane, Frenchay, Bristol, BS16 1QY

The neuropeptide proctolin (ARG-TYR-LEU-PRO-THR) has been found in the nervous tissue of some insects [1]. In addition, proctolin has been shown [2] to be a powerful stimulant of the hind gut of the locust *Locusta migratoria* while we [3] have shown that proctolin causes contraction of the isolated foregut of *Schistocerca gregaria*. The aim of this study was to compare the effects of exogenously applied proctolin on the isolated fore- and hind-guts of the latter species in the presence and absence of a range of antagonists and calcium channel blockers.

Isolated foreguts (oesophagus to proventriculus) and hindguts of *Schistocerca gregaria* were incubated in Clark Insect Ringer at room temperature (18 ± 2°C) for 20 min, and using a 6 min cycle with two washes, dose response curves were constructed for proctolin (1 - 10000 nM). The effects of mianserin (50 μM), ketanserin (50 μM), kynurenic acid (10 μM), picrotoxinin (1 μM) and d-tubocurarine (10 μM) on the response of the foregut to proctolin were investigated with the tissue being incubated with an antagonist for 20 min prior to retesting the effect of the peptide. However, the response of both tissues to proctolin was challenged by the addition of either caffeine (1 - 100 μM), verapamil (1 - 100 μM) or cadmium ions (50 - 500 μM) using an identical regime to that described above.

Proctolin caused dose dependent contraction of the fore (10 - 1000 nM; ED_{50} : 160 ± 30 nM; n = 6) and hind gut (1 - 500 nM; ED_{50} : 17.5 ± 1.6 nM; n = 7) showing marked tachyphylaxis at higher doses. These responses were unaffected by the presence of mianserin, ketanserin, kynurenic acid, picrotoxinin, d-tubocurarine and caffeine. However, verapamil (100 μM) and cadmium ions (100 μM) significantly reduced (60 - 70%) proctolin-induced contraction of both tissues. Cadmium ions at concentrations in excess of 300 μM totally abolished the spasmogenic effect of proctolin.

The hindgut of *Schistocerca gregaria* proved to be significantly more sensitive to the effects of applied proctolin than the foregut which required a ten fold higher dose of the agonist cause a half maximal contraction. The ED_{50} value for the hindgut (17.5 nM) is comaparable to that reported [4] for the hindgut of the cockroach *Periplaneta americana*.

It has been suggested that proctolin is a co-transmitter of 5-hydroxytryptamine (5-HT) [5] and a neuromodulator of glutamate [6] and octopamine [7]. However, the failure of mianserin, ketanserin, kynurenic acid, d-tubocurarine and picrotoxinin to inhibit proctolin-induced contraction of the foregut suggests that this effect is not mediated via octopamine (OA), $5\text{-}HT_2$, glutamate, GABA or nicotinic acetylcholine

receptors respectively. Furthermore, as 5-HT and GA cause relaxation of foregut and glutamate is but a weak spasmogenic agent [3] it would appear to be unlikely that the contractile effects of proctolin are mediated by an interaction with these substances thereby suggesting the presence of a specific proctolin receptor in the tissue.

With regard to secondary messengers mediating the effects of proctolin, the lack of effect of the phosphodiesterase inhibitor caffeine suggests that the receptor is not linked to adenylate cyclase. However, verapamil and cadmium ions strongly antagonized the contractile effect of the peptide thereby suggesting that calcium ions may act as the secondary messenger.

1. Brown, B.E. J. Insect Physiol. 1977 23 861 - 864

2. Dunbar, S.J. and Piek, T. Arch. Insect Biochem. Physiol. 1977 1 93 - 104

3. Banner, S.E., Osborne, R.H. and Cattell, K.J. Comp. Biochem. Physiol. 1987 88C 139 - 144

4. Sullivan, R.E. and Newcomb, R.W. Peptides 1982 3 337 - 344

5. Siwicki, K.K., Beltz, B.S. and Kravitz, E.A. J. Neurosci. 1987 7 522 - 532

6. Cook, B.J. and Holman, G.M. Comp. Biochem. Physiol. 1979 64C 183 - 190

7. Orchard, I. and Lange, A.B. J. Insect Physiol. 1987 33 265 - 268

THE ACTIONS OF FMRFamide-LIKE PEPTIDES ON LOCUST HEART

Brian A. Cuthbert and Peter D. Evans

AFRC Unit, Dept of Zoology, University of Cambridge
Downing Street, Cambridge, CB2 3EJ

FMRFamide (Phe-Met-Arg-Phe-NH_2), a neuropeptide first isolated from the clam Macrocallista nimbosa[1], and some of the structurally related family of FMRFamide-like peptides discovered subsequently in a range of species, have modulatory effects on invertebrate skeletal and visceral muscle. Immunocytochemical evidence suggests that FMRFamide-like peptides are present in specific subsets of neurones in the nervous systems of various vertebrate and invertebrate species. In the locust Schistocerca gregaria FMRFamide-like immunoreactivity has been located in the ganglia of the ventral nerve cord[2,3]. Some of the FMRFamide-immunoreactive cells have processes which terminate in the neurohaemal organs of the median nerves of the thoracic ganglia. Recently, extracts of the neurohaemal organs of the metathoracic ganglia in Schistocerca have been shown to mimic the modulatory actions of FMRFamide on locust skeletal muscle[4]. It is therefore possible that FMRFamide-like peptides are released into the haemolymph as circulating neurohormones.

Although locust skeletal muscle may be an important target site for the actions of FMRFamide-like peptides[2,3], these peptides also have effects on myogenic rhythm in visceral tissues. In many invertebrates such as molluscs and the leech [5,6] FMRFamide-like peptides are cardioactive and this was also found to be the case in Schistocerca[7].

A large range of the structurally-related FMRFamide family were tested on a semi-isolated locust heart preparation, to determine the structural requirements of the receptors sensitive to FMRFamide-like peptides. FMRFamide regularises the heart beat of the locust and increases the frequency and strength of contractions in a dose dependant manner. From the range of FMRFamide peptide analogues tested a variety of criteria can be proposed for the structure specificity of the receptors on the heart.

1. C-terminal amidation is essential since all those peptides tested which lacked the -NH_2 group failed to elicit a response.

activity, although the peptide Arg-Phe-NH_2 is in itself not sufficient for activation of the receptors.

3. Methionine or Leucine preceeding the -Arg-Phe-NH_2 sequence does confer activity, though replacing either of these with another amino-acid such as Proline in FPRFamide renders the peptide inactive on the heart.

4. N-terminal extention of the basic tetrapeptide F(M/L)RFamide increases the potency 10 - 100 times. However, the extended peptides caused inhibition of contractions at concentrations above 10^{-7}M.

5. With YGGFMRFamide there is an added complication: at concentrations below 10^{-7}M the peptide is excitatory, however above this the initial excitation of the heart is followed by an inhibition which continues for the duration of the pulse. Once the pulse of YGGFMRFamide ends the excitatory response is restored.

The structural requirements for the receptors on the heart seem to be similar to FMRFamide-like peptide receptors in other systems studied such as in the locust extensor tibiae muscle[8], and various molluscan cardiac tissues. Similarly the responses to N-terminally extended peptides are comparable. However, the biphasic response evoked by YGGFMRFamide suggests that there are perhaps two types of receptors on the heart mediating the actions of FMRFamide-like peptides in the locust heart. One receptor can increase the frequency of contractions and is activated by FMRFamide and low doses of YGGFMRFamide, and the other can reduce frequency of contractions and is activated by high doses of YGGFMRFamide.

1. Price, D.M. & Greenberg, M.J. Science 1977 197 670-671
2. Myers, C.M. & Evans, P.D. J. Comp. Neurol. 1985 234 1-16
3. Myers, C.M. & Evans, P.D. Cell Tissue Res. 1985 242 109-114
4. Walther, C. & Schiebe, M. Neurosci. Letts. 1987 77 209-214
5. Painter, S.D. & Greenberg, M.J. Biol. Bull. 1982 162 311-332
6. Kuhlman J.R., Li, C. & Calabrese, R.L. J. Neurosci. 1985 5 2310-2317
7. Myers, C.M. PhD Thesis, chap 6. Cambridge University 1986
8. Evans, P.D. & Myers, C.M. J. exp. Biol. 1986 126 403-422

PRODUCTION OF SNAKE AND SCORPION VENOMS CONTAINING NEUROTOXINS

Yvon DOLJANSKY, Yves MITTAINE, Anne-Marie de SAINT-MICHEL
LATOXAN (Laboratoire des Toxines Animales et Animaux Venimeux)
F 05150 ROSANS, France

To protect themselves from predators, or to immobilize swiftly their own preys, various animals produce sophisticated and extremely active biological weapons : VENOMS.

It is known that a number of snake and scorpion venoms contain neurotoxins, whose major modes of action can be classified as follows :

- Acetylcholine receptor blocking neurotoxins
- Acetylcholine release facilitatory neurotoxins
- Post-synaptic blocking neurotoxins
- Acetylcholinesterase inhibitors
- Ion channels (K^+ ; Na^+) marking neurotoxins, e.g. "Charybdotoxins" which are known to block single Ca^{2+}-activated K^+ channels.

Another class of neurotoxins can be added to this list : the insect-specific neurotoxins ("insect-toxins") which are found in most scorpion venoms.

A few laboratories initiated research on snake and scorpion venom neurotoxins more than twenty years ago, and interest spread widely in the past 5 years. To-day highly resolutive purification procedures are available, which enable to obtain extremely pure neurotoxic components. However, most venoms are still to be explored further, their modes of action and possible applications remaining quite unelucidated.

LATOXAN is a leading venom production center for biological research worldwide. Its productions are based on an unequalled stock of animals brought into the laboratory from all parts of the world, or born and reared in the laboratory : more than 1,000 snakes and several thousand scorpions are permanently and carefully maintained. They represent more than 100 different species and sub-species.

Production procedures ensure :

- full control of venom identification and purity ;
- the highest biological activities of the venoms ;
- continuity of production over the years (a condition for reproducible research results).

All modes of action listed above can be obtained from neurotoxins which are present in various venoms among those currently produced by LATOXAN.

EFFECTS OF SCORPION TOXIN 2 FROM ANDROCTONUS AUSTRALIS HECTOR ON ELECTRICAL ACTIVITY IN FROG AND RAT SKELETAL MUSCLES.

Alain Duval*, Marcel Pelhate* and Marie-France Martin**

* Department of Neurophysiology, UA CNRS n° 611, The University of Angers, 49045 Angers Cedex, France.
** Department of Biochemistry, U.D.C., CNRS UA 1179 - INSERM U 172, Medical School, 13326 Marseille Cedex, France.

The alpha toxins[1] extracted from various species of scorpions are known to prolong the falling phase of the action potential by affecting the inactivation of the Na conductance in excitable membranes. However, because most studies have been performed on various nerve preparations (see Schmidtmayer[2], Benoit et Dubois[3]) only very little[4] is known about the effects of these compounds on skeletal muscle electrical activity. We have therefore investigated the action of the mammal toxin 2 from Androctonus australis Hector[5] (MT_2 AaH) on rat (fast EDL and slow Soleus twitch muscles) and frog (semitendinosus muscle) skeletal muscle fibres. The toxin effects and its relative potency in rat and frog muscles have been determined by studying the modifications in action potential shape and Na current properties measured under current and voltage clamp conditions, with the double sucrose-gap technique[5].

Under current clamp conditions, the evoked action potentials were prolonged by MT_2 AaH (from their usual duration, 5 - 10 msec at room temperature, to hundreds of milliseconds or even seconds, depending on the toxin concentration 10^{-10} - 10^{-6}M) without significant effects on their rate of rise and amplitude. The resting potential was unchanged in the range 10^{-10} to 10^{-5} M. Both EDL and Soleus fibres of rat were more sensitive to the toxin than frog fibres. The effects on action potential duration were half maximum at toxin concentration of 1.4×10^{-9} M for rat and 9×10^{-9} M for frog muscles, and the maximum effects obtained were more important in rat than in frog (plateau action potential durations = 5 and 1,5 seconds respectively). The action potential prolongation was reduced by repetitive stimulation (0,01 - 10 Hz). ...

Under voltage clamp conditions MT_2 AaH slowed the Na current inactivation process and induced a non-inactivatable Na current. Peak and maintained currents were TTX-sensitive and reversed at the same potential. The amplitude, activation time course and current-voltage relationship of the peak Na current were not significantly altered by the toxin. The amplitude of the maintained Na current was dependent on toxin concentration, reaching a maximum value representing 15% and 30% of the peak current in rat and frog muscles respectively. The half maximum effects for this parameter was obtained at toxin concentrations of 0.8×10^{-9} M in rat and 15.5×10^{-9} M in frog fibres. The maintained Na permeability had a different voltage dependence than that of peak Na permeability (half - maximum value 20 mV more positive). In the presence of MT_2 AaH the reactivation and steady state inactivation of the inactivating Na current (i.e., peak current minus maintained current) remained unchanged. The action of the toxin reversed poorly by washing out but could be largely removed by conditioning depolarizations more positive than the reversal potential of the Na current. Even at 10^{-5} M MT_2 AaH did not affect the K currents.

The results indicate that MT_2 AaH acts on vertebrate muscle fibres like others alpha toxins on nerve membrane[2, 3] by modifying the inactivation of the Na channels. Moreover they reveal a higher specificity of MT_2 AaH toward mammals than toward amphibians. By comparison with previous results[7] MT_2 AaH seems to be much more potent in vertebrates than in insects.

1. Jover, E., Couraud, F. and Rochat, H. Biochem. Biophys. Res. Commun. 1980 95 1607-1613
2. Schmidtmayer, J. Pflügers Archiv 1985 404 21-28
3. Benoit, E. and Dubois, J.M. J. Physiol. 1987 383 93-114
4. Catterall, W.A. J. Gen. Physiol. 1979 74 375-391
5. Miranda, F., Kopeyan, C., Rochat, C., Rochat, H. and Lissitzky S., Eur. J. Biochem. 1970 16 514-523
6. Duval, A. and Leoty, C. J. Physiol. 1978 278 403-423
7. Pelhate, M. and Zlotkin, E. J. exp. Biol. 1982 97 67-77.

THE EFFECT OF AZADIRACHTIN ON THE LARVAE OF THE CERAMBYCID <u>MORIMUS FUNEREUS</u>

Marina B. Frusic, Jelisaveta P. Ivanovic, Miroslava I. Jankovic-Hladni and Vlasta O. Stanic.

Institute for Biological Research "Sinisa Stankovic", 29. novembra 142, 11000 Belgrade, Yugoslavia

Much attention has recently been paid to studies on the effect of azadirachtin on insects[1,2,3,4] which results are expected to be useful for the control of harmful insects, but little work has been done to clarify the mechanisms of azadirachtin action on insects.

In the scope of our investigations on the response of the larvae of the cerambycid <u>Morimus</u> <u>funereus</u> on the effect of stressors studied at different levels of biological organization, the effect of azadirachtin[5] as a stressor was included into our studies.

Larvae of <u>M</u>. <u>funereus</u>, reared on a synthetic diet at 23°C in darkness, were used in experiments 0-24 h following ecdysis. They were injected with 5 μg and 10 μg of azadirachtin (per 1 g body weight) dissolved in 10% ethanol and with the same amount of 10% ethanol (control II); the intact larvae served as control I. The following parameters were followed comparatively: the body weight, food intake, morphogenetic changes as well as some biochemical parameters proved to be under the control of neurohormones (acti-

vity of midgut amylase and protease, concentration of fat body glycogen and haemolymph trehalose).

The results show that two weeks after the treatment, lower concentration of azadirachtin had no effect on the body weight of the larvae but it provoked appearance of small dark patches on the integument which increased in size in the course of time. Higher concentration of azadirachtin affected the body weight of larvae i.e. two weeks after the treatment the larvae exhibited a slight decrease of their initial weight, while those injected with ethanol were similar to the intact larvae.

All the results obtained will be discussed from the aspect of the effect of azadirachtin on the insect neuroendocrine system.

1. Barnby, M.A. and Klocke, J.A. J. Insect Physiol. 1987 33 69-75
2. Dorn, A., Rademacher, J.M. and Sehn, E. J. Insect. Physiol. 1986 32 231-238
3. Koul, O., Amanai, K. and Ohtaki, T. J. Insect Physiol. 1987 33 103-108
4. Rembold, H., Sharma, G.K., Czoppelt, Ch. and Schmutterer, H. Z. ang. Ent. 1982 93 12-17

5. Dr M. Barnby (Salt Lake City, Utah, USA) kindly provided azadirachtin; this research was sponsored by the Republic Scientific Community of Serbia (RZN) and the USDA Project Nº JB-129 JFP 657.

PUMILIOTOXIN B BINDS TO A NOVEL SITE ON THE VOLTAGE-DEPENDENT SODIUM CHANNEL THAT IS ALLOSTERICALLY COUPLED TO OTHER BINDING SITES FOR PEPTIDE TOXINS.

Fabian Gusovsky, Daniel Rossignol*, Elizabeth T. McNeal and John W.Daly

Laboratory of Bioorganic Chemistry, NIDDK, National Institutes of Health, Bethesda, MD 20892 and *E.I. du Pont de Nemours & Company, Experimental Station, Wilmington, DE 19898, USA.

The alkaloid pumiliotoxin B (PTX-B), isolated from the skin of the neotropical frog Dendrobates pumilio, has myotonic[1] and cardiotonic[2] activity and induces repetitive firing of action potentials in nerve and muscle[3]. In brain synaptoneurosomes, PTX-B elicits a breakdown of phosphoinositides that is dependent on the presence of sodium and that can be blocked with the sodium channel blocker tetrodotoxin[4]. The possible actions of PTX-B on the voltage-dependent sodium channels have now been investigated by studying the effects on ^{22}Na influx in synaptoneurosomes and neuroblastoma cells.

PTX-B induces a small dose-dependent influx of sodium in synaptoneurosomes which is markedly enhanced in the presence of α-scorpion toxin (α-ScTx) or a scorpion venom (Leiurus) (ScV) that contains α-ScTx. In N18 neuroblastoma cells, PTX-B is inactive on sodium fluxes alone but in the presence of α-ScTx, PTX-B stimulates sodium flux to levels comparable to that attained with the sodium channel activator veratridine. Similar to other channel activators, the influx of sodium caused by PTX-B in combination with α-ScTx or ScV can be antagonized by local anesthetics and tetrodotoxin in both synaptoneurosomes and N18 cells. In LV9 neuroblatoma cells, a variant mutant that lacks

sodium channels, PTX-B and veratridine, either in the presence or the absence of ScV, were unable to stimulate influx of sodium. PTX-B does not interact with two known binding sites on the sodium channel, as evidenced by its lack of effect on binding of [^{3}H]saxitoxin and [^{3}H]batrachotoxinin A benzoate in brain synaptoneurosomes. In addition to α-ScTx, PTX-B induces synergistic responses on sodium flux in synaptoneurosomes when combined with β-scorpion toxin and with the polyether brevetoxin. Thus, PTX-B apparently does not act through binding at known toxin sites on the sodium channel but instead it induces sodium flux throgh the interaction with yet another modulatory site on the channel. The allosteric associations with α-ScTx, β-scorpion toxin and brevetoxin suggest that the PTX-B site occupies a pivotal position on the gating modulation of the sodium channel.

1. Albuquerque, E.X., Warnick, J.E., Maleque, M.A., Kaufman, F.C., Tamburini, R., Nimit, Y. and Daly, J.W. Mol. Pharmacol. 1981 19 411-424

2. Daly, J.W., McNeal, E.T., Overman, L.E. and Ellison, D.H. J. Med. Chem. 1985 28 482-486

3. Rao, K.S., Warnick, J.E., Daly, J.W. and Albuquerque, E.X. J. Pharmacol. Exper. Ther. 1987 243 775-783

4. Gusovsky, F., Hollingsworth, E.B. and Daly, J.W. Proc. Natl. Acad. Sci. USA 1986 83 3003-3007

EFFECTS OF THR^{6} BRADYKININ ON SYNAPTIC TRANSMISSION IN THE INSECT CNS

Bernard Hue[1] and Tom Piek[2]

[1]Department of Neurophysiology, UA CNRS no. 611, The University of Angers, 49045 Angers Cedex, France and [2] Department of Pharmacology, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands.

Europe's largest wasp, <u>Megascolia flavifrons</u> stings beetle larvae in the direction of the nerve ganglia[1]. The effect on the insect CNS, of the venom, one of its fractions, and Thr^{6} bradykinin isolated from this fraction[2], was studied using a preparation of the desheated sixth abdominal ganglion of the cockroach in connection to the cercal nerve XI and to the connective, or its isolated giant axons between ganglion VI and V. The venom and the Thr^{6} bradykinin containing fraction cause a block of synaptic transmission from the cercal nerve to the giant interneuron, and a delayed depolarization of the neuron. Thr^{6} bradykinin only causes a slow and delayed decrease in EPSP and unitary potential amplitude and has no effect on carbachol potentials, and membrane conductance and excitability of the giant neuron.

It can be concluded that Thr^{6} bradykinin blocks the transmission presynaptically, probably by depletion (Fig. 1).

References

1. Piek, T., Buitenhuis, A. Simonthomas, R.T., Ufkes, J.G.R. and Mantel, P. Comp.Biochem.Physiol. 1983. 75C, 145-152.
2. Yasuhara, T., Mantel, P., Nakajima, T. and Piek, T. Toxicon 1987. 25, 527-535.

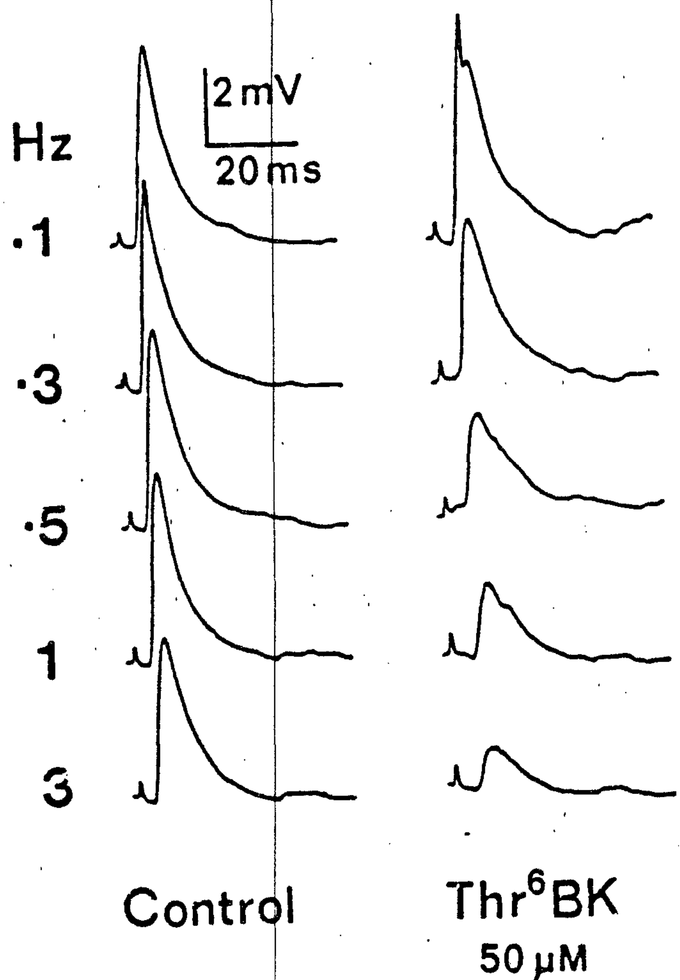


Fig. 1. Effects of the stimulus frequency on steady-state EPSP amplitudes recorded from a giant neuron in the sixth abdominal ganglion of the cockroach, during control, and in the presence of Thr^6 bradykinin.

THE PROTHORACIC GLAND ACTIVITIES DURING THE LAST LARVAL INSTAR OF LEPIDOPTEROUS INSECTS

Mamdouh H. Idriss

Division of Entomology, Faculty of Agriculture, Alexandria University, Alexandria, Egypt

After the pioneering experiments of Kopeć[1] (1917), and the findings of Fukuda[2] (1940), Williams[3] (1946) proved the role of prothoracic gland (PTG) in moulting process. The active PTGs are able to induce moulting in isolated abdomen whereas an active brain can only produce the same effect in the presence of inactive gland. Although several comparativ morphological studies of the PTG have shown that lepidopterous' PTGs are richly supplied with nerves, the experimental evidence proves the necessity of an intact cerebral neurosecratory system for normal moulting. There is no doubt that the prothoracicotropic hormone (PTTH) is considered a putative factor affecting PTG but the hormonal relationship between an insect brain and PTG has not yet been well defined. When whole brains are implanted immediately into individuals whose own neurosecretory cells have been removed, moulting is delayed compared with normal insects. During the present study the correlation between the morphological changes of PTG and its physiological activities was investigated. Furthermore we attempt to provide an introduction to other factors involved with PTG activation.

The fifth instar larvae of Eri-silkworm *Philosamia ricini* (Dru.), as a well established model system in our lab, were used for the present investigation. The PTTH bioassay test-organism which was reported previously[4], has been improved. We produced brainless pupae from debrainated larvae. After the critical period of PTTH[5], *Ph. ricini* larvae were debrainated. These larvae, almost two weeks after the surgical operation, moulted to brainless pupae. They were reliable bioassay animals for detecting prothoracicotropin-like effects. The critical period of moulting hormone (PTGH) was determine using the isolated abdominae technique. To prepare isolated abdomen, a *Ph. ricini* larva was ligated in front of the third abdominal segment. The anterior portion of the larva was removed and wound was sealed with gelatine and antibiotics.

Scanning electron micrograph (A) shows the metamorphic endocrine centres of a caterpillar: (1) brain, (2) corpus paracardiacum CC, (3) corpus allatum CA and (4) PTG. The prothoracic gland receives its nerve supply from the ventral sympathetic system which arise from the suboesophageal and segmental ganglia. Scanning electron micrograph (B) illustrates the central part of PTG inervation. The role of these neuroglandular junctions and its neurotransmitter(s) still unkown.

The PTGH critical-period of *Ph. ricini* larvae was experimentally determined using isolated-abdominae techique. Abdominae were isolated at different times (0, 24, 48, 72, 96, 120 & 144 hrs after the beginning of cocoon spinning). These isolated abdominae were observed and percentage of pupation for each group was recorded. As shown in (C) PTGH critical-period is 81.11 hrs after the beginning of cocoon spinning. In the light of the above mentioned data, histological studies of PTG were investigated. Light micrographs D,E,F&G show the cytological activities of the gland immediatly before conoon spinning, 72, 96& 120 hrs after the beginning of cocoon spinning respectively. It seems clearly that the histological studies confirm the experimental data concerning PTGH critical-period.

According to our knowledge of PTTH critical-period, the debraination of *Ph. ricini* larvae immediatly before cocoon spinning produced brainless pupae. The normal pupation occures after 5±0.57 days, however, debrainated larvae pupated after 16.73±0.27 days with 24.4% larval-pupal intermediates. Injection of debrainated larvae with epinephrine (0.05 μg/L) decreased abnormalities to 17.3% while juvenile hormone analog ZR-619 (5 μg/L) increased deformations to 52.8% Epinephrine and JHA accelerated the pupation. Debrainated larvae injected with epinephrine or JHA pupated after 15.56±0.21 days and 14.79±0.13 days

respectively. It should be mentioned that 14.7% of the brainless pupae emerged to brainless moths after 32.6±0.8 days. So the reliability of this bioassay organism is 31 days after pupation.

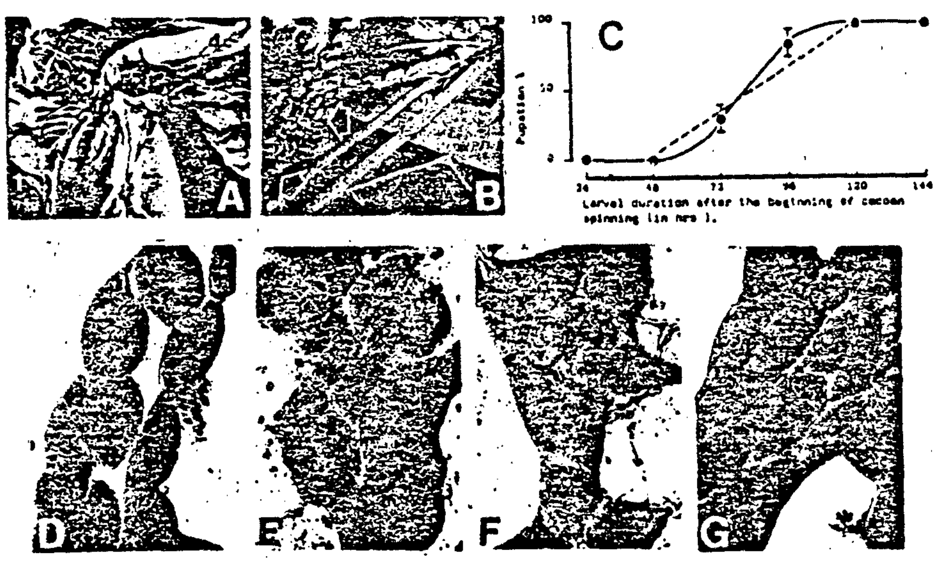
The injection of debrainated larvae with 0.05 µg/L epinephrine or 5 µg/L ZR-619 increased the percentage of moths emerged from brainless pupae to 31.6% after 27.5±1.1 days for catecolamine and to 20% after 22.6±0.7 days for JHA. The emerged moths produced after JHA treatment were completly deformed.

Beta ecdyson (PTGH) was injected at different concentrations into brainless *Ph. ricini* pupae. As it was expected, brainless moths emerged from the brainless pupae which were injected with 0.5-10 µg ecdyson. Indeed it was surprising enough that the injection with high concentrations of ecdyson (>50 µg/P) produced pupal-adult intermediates. This case was not pseudojuvenilization. On the other hand Idriss *et al*[6] proved that some of catecolamines had a porthoracicotropin-like effects on decapitated *Ph. ricini* larvae. When these compounds were tested on the brainless *Ph. ricini* pupae they didn't show any effect.

In general, the critical period of PTGH could be determined using either histological techniques or an experimental method such as isolated-abdominae technique. It must, however, be realized that the histometric method could not be adopted to study the activity of PTG.

From the results obtained after injection ecdyson and JHA into brainless *Ph. ricini* pupae, it seems that ecdyson at high concentrations activates CA, on the other hand JHs activate PTG. It is reasonable to presume that there is a positive feedback-mechanism controlling the relationship between PTG and CA under certain conditions.

Finally, if we assume that PTG neuroglandular junctions are catecolaminergic synapses, there is a clear evidence that catecolamines have a secondary role during PTG activation.



1- Kopeć, S. 1917. *Bull. int. Acad. Sci. Cracovie* (B), pp 57-60.
2- Fukuda, S. 1940. *Proc. imp. Acad. Japan*, 16, 417-20.
3- Williams, C.M. 1946. *Biol. Bull., Woods Hole*, 90, 234-243.
4- Idriss, M., Elgayar, F. & Rawash, I. 1981. Z. ang. Ent. 92, 371-374.
5- Idriss, M. 1986. *6th Inter. Cong. Pesti. Chem.* Canada 2B-10.
6- Idriss, M., Sherby, S., Morshedy, M.& Mansour, N. 1984. in *Insect Neurochem. & Neurophysiol.* (Borkovec, A.& Kelly, T. eds.) Plenum Press, NY 385-387.

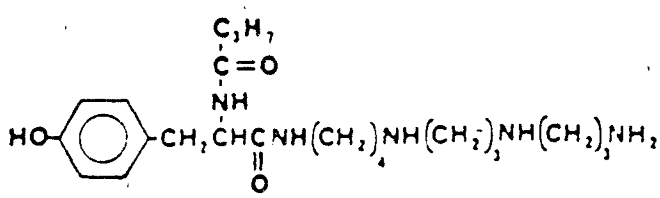
BLOCK OF TRANSMISSION AND GLUTAMATE REUPTAKE INHIBITION IN INSECT MUSCLE BY SYNTHETIC DELTA-PHILANTHOTOXIN

Henk Karst, Albert Lind, Jan van Marle*, Tom Piek and Janneke van Weeren-Kramer

Departments of Pharmacology and Electronmicroscopy*, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

The wasp Philanthus triangulum paralyses workers of the honeybee. The venom is active in all insects, and the excitatory glutamatergic neuromuscular transmission is affected in two different ways: by a presynaptic inhibition of the reuptake of glutamate[1,2], and by a postsynaptic block of open ion channels[3,4].

Spectroscopic evidence strongly points to a structure for delta-philanthotoxin, isolated from this venom, as follows[1]:



The synthesis of this toxin has confirmed the correctness of this proposal. NMR-spectra of both the natural δ-PTX and the synthetic toxin, called δ-PTX-S_1, are identical[1]. The polyamine chain is thermospermine.

EM-experiments were carried out with isolated retractor unguis muscles of the hindleg of the locust Schistocerca gregaria. Six control muscles were preincubated in saline for 10 min, subsequently incubated for 60 min in saline containing $[^3H]$glutamate (4.3×10^{-4} M, 100 μCi/ml) and afterwards rinsed in saline 2 x

10 min at 0°C. Six other muscles were pretreated in 52 µg/ml δ-PTX-S_1, and treated as described before, in the presence of the toxin. EM-autoradiographs were prepared using the flat substrate method[2,3]. The average grain densities over both terminal axons and glial-cells show a reduction of glutamate uptake of more than 50% (Table 1). Glutamate potentials of concavalin A pretreated locust muscles were reversibly curtailed and blocked by the toxin at a concentration of about 10^{-7} M.

Table 1. Grain densities (expressed as the numbers of developed silver grains per μm^2; average values ± SEM, n=4) over both terminal axons TA and their surrounding glial cells (GI) of retractor unguis muscles incubated with δ-PTX-S_1, compared with the control.

	TA	GI
Control	16.4 ± 1.2	46.0 ± 3.7
δ-PTX-S_1	7.7 ± 1.8	17.9 ± 2.8

References

1. Piek, T., Karst, H., Kruk, C., Lind, A., Nakajima, T., Nibbering, N.M.M., Shinozaki, H., Spanjer, W., Tong, Y.C. and van Marle, J. Abstract of this meeting 1988.
2. Kopriwa, B.M. Histochemistry 1973, 37, 1-17.
3. Van Marle, J., Piek, T., Lind A. and Van Weeren-Kramer, J., Comp.Biochem.Physiol. 1983, 74C, 191-194.

EFFECTS OF SPIDER TOXINS ON GLUTAMATE TRANSMISSION AND UPTAKE

Henk Karst, Albert Lind, Jan van Marle*, Terumi Nakajima+, Tom Piek and Janneke van Weeren-Kramer

Departments of Pharmacology and Electronmicroscopy*, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands and +Faculty of Pharmaceutical Sciences, Bunkyo-ku, Tokyo 113, Japan.

Spider toxins: JSTX-3 from the venom of the Japanese spider, Nephila clavata, and NSTX-3 from the New Guinea's spider Nephila maculata, are neurotoxins that block irreversibly the excitatory glutamatergic neuromuscular transmission in the lobster leg[1-3]. In crustacean muscle JSTX-3 suppressed the EPSP's at a concentration of $2 \times 10^{-[illegible]}$ M and no recovery was found by washing the preparation for more than 2h[illegible].

We have tested both toxins on the excitatory neuromuscular synaps of the locust (Schistocerca gregaria) skeletal muscle at two different ways: (1) studying the iontophoretically evoked glutamate potential, using muscles with and without pretreatment by concanavalin A and (2) using EM-autoradiography to study the high affinity glutamate uptake[illegible].

At concentrations varying from $(1.5\text{-}4.5) \times 10^{-[illegible]}$ M both toxins block glutamate potentials. The effect is reversible, however, more slowly than seen with the wasp toxin δ-PTX (this meeting). The speed of recovery of the block by NSTX-3 is highly increased by pretreatment of the preparation with concanavalin A ($2 \times 10^{-[illegible]}$ M).

Comparison of photographs of toxin treated terminal axons and glial cells with controls did not reveal any morphological differences. No accumulation of synaptic vesicles was observed. Nor did the muscles show any kind of degenerative changes. However, analysis of the grain densities above the terminal axons and glia revealed that both spider toxins significantly increased the glutamate uptake by terminal axons and glia (Table 1).

Table 1. Grain densities over terminal axons (TA) and their surrounding glia cells (Gl) of retractor unguis muscles incubated with NSTX-3 or JSTX-3 at a concentration of 100 μM. The densities are expressed as the numbers of developed silver grains per μm^2; (average values ± SEM n=4).

	TA	Gl
control	8.1 ± 1.0	29.8 ± 4.0
NSTX-3	17.7 ± 2.5	45.5 ± 5.3
JSTX-3	15.3 ± 1.3	46.8 ± 1.7

References

1. Kawai, N., Miwa, A. and Abe, T. Brain Res. 1982, 247, 169-171.
2. Kawai, N., Miwa, A. and Abe, T. Biomedical Res. 1982, 3, 353-355.
3. Nakajima, T. and Kawai, N. Abstr. 1st Asia-Pacific Congr.Anim., Plant Microb.Toxins, Singapore, 1987, 0059.
4. Shudo, K., Endo, Y., Hashimoto, Y., Aramaki, Y., Nakajima, T., and Kawai, N. Neurosc.Res. 1987, in pres.
5. Van Marle, J., Piek, T., Lind, A. and Van Weeren-Kramer, J. Comp.Biochem.Physiol. 1983, 74C, 191-194.

The mode of action of locust hypolipaemic hormone.

By

B.G. Loughton
Biology Department
York University
North York, Ontario
Canada

The hypolipaemic hormone of the locust acts to decrease haemolymph lipid levels after excitation, flight or feeding (Loughton 1987). Application of this hormone in crude or partially purified form to intact locusts or to locust fatbody *in vitro* resulted in a substantial increase in fatbody cyclic AMP. Bovine insulin mimicked this effect. In addition injection of forskolin, an activator of adenylate cyclase, mimicked the action of hypolipaemic hormone *in vivo*. These results were surprising since it has been claimed that adipokinetic hormone (AKHI) exerts its lipid mobilizing effect on the fatbody by activating adenylate cyclase.

Injection of physiological doses of synthetic AKHI into locust did not result in an elevation of cAMP in the fatbody. Treatment of locust fatbody *in vitro* with similar doses did increase fatbody cAMP.

In order to investigate this phenomenon further crude membrane fractions from locust fatbody were incubated in an ATP regenerating system *in vitro* together with a range of test substances. Significant increases in cAMP were observed after treatment with hypolipaemic hormone and the insulin molecule. No increase was observed after incubation with AKHI.

It is concluded that locust hypolipaemic hormone activates fatbody adenylate cyclase and that AKHI does not.

STUDIES ON OVICIDAL PROPERTIES OF SOME NATURAL PRODUCTS ON THE EGGS OF _HENOSEPILACHNA VIGINTIOCTOPUNCTATA_ FABR.

Prem N. Mishra

Hill Campus, G.B. Pant Univ. Of Agric. & Tech. Ranichauri(Tehri) U.P. INDIA- 249 199

Black pepper (_Piper nigrum_ Linn.), fennel (_Foenicum vulgare_ Mill.), chamomille (_Matricaria chamomilla_ Linn.), lupin (_Lupinus termis_ Fersk), cumin (_Cuminum cyminum_ Linn.), dharek (_Melia azedarach_ Linn.) and garlic (_Allium sativum_ Linn.) have been reported [1-4] to be toxic for various insect pests. These plant products (5 & 10% cold aqueous extracts) were assessed for their ovicidal action on the freshly oviposited eggs of brinjal leaf beetle, _H. vigintioctopunctata_. Dharek (_M. azedarach_) proved most effective ovicide inflicting 76.67 and 91.66 per cent egg mortality in 5 and 10 per cent concentrations, respectively. Garlic extracts ranked next effective ovicide inflicting 40.0 and 60.0 per cent egg mortality in the respective concentration. Other plant products proved weakest ovicides and found significantly at par with control in-respect to per cent egg mortality. Meliantriol a major active principle has been reported[5] in the drupes of _M.azedarach_ which is a systemic triterpenoid compound and might be responsible for this marked ovicidal action. Diallyl disulphide and Diallyl trisulphide present in the garlic extract might be responsible for this observed higher toxicity for the eggs of _H. vigintioctopunctata_.

1. Atwal, A.S. and Pajni, H.R. Indian J. Ent. 1964 26 221
2. Barakat, A.A., Fahmy, H.S.M., Kandil, M.A. and Nabil, M.M. Indian J. agric. Sci. 1985 55 116
3. Sandhu, G.S. and Singh, D. Indian J. Pl. Prot. 1975 3 177
4. Teotia, T.P.S. and Tewari, G.C. Indian J. Ent. 1971 39 222
5. Lavie, D., Jain, M.K. and Gabrielith, S.R.S. Chem. Commun. 1967 910

Mordue (Luntz), A.J. & Plane, F.

Muscular effects of azadirachtin

Azadirachtin, a complex triterpenoid from the neem tree *Azadirachta indica*, produces marked growth and moulting abnormalities in a very large number of insect species. Investigations on its toxicology in *Locusta migratoria* have revealed direct effects on gut muscle. Work is now in progress to identify the lesion which is seen on proctolin- but not glutamate-stimulated hind guts.

KININS ISOLATED FROM THE VENOM OF THE SOLITARY WASP MEGASCOLIA FLAVIFRONS

Terumi Nakajima[1], Tom Piek[2], Tadashi Yasuhara[1] and Piet Mantel[2]

[1] Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan and [2] Department of Pharmacology, Meibergdreef 15 1105 AZ Amsterdam, The Netherlands.

Larvae of the beetle Oryctes nasicornis are paralysed by stings of the wasp M.flavifrons[1]. The venom contains histamine and a bradykinin-like substance[1,2]. Recently it has been found, that the venom and some of the fractions block synaptic transmission in the cockroach sixth abdominal ganglion[3].

Females of this wasp were collected in the south of France, and killed and stored in liquid nitrogen. Venom reservoirs were removed from thawed wasps and extracted in distilled water. Freeze dried extracts were dissolved in 5% acetonitrile in 0.2% HCl. The supernatant was fractionated by HPLC using a linear gradient from CH_3CN: 0.05% CH_3COOH, 5:95 to CH_3CN:0.05% CH_3COOH, 3:7. Each of the 33 fractions (Fig. 1) was tested using a cascade of smooth muscle preparations, indicating bradykinin-like activity in the fractions 9 and 14. Rechromatography of these fractions using reserve phase columns (ODS LS-120-T, Toyo Soda) with isocratic elution (CH_3CN:0.02% HCl, 225:77.5 for fraction 14 and 22.7-117.5 for fraction 9, was followed by amino acid analysis.
Fraction 14 contained

Arg-Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg
(Thr^{6} bradykinin)

Fraction 9 contained

Arg-Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg-Lys-Ala
(Thr^{6} bradykinin-Lys-Ala, or megascoliakinin)

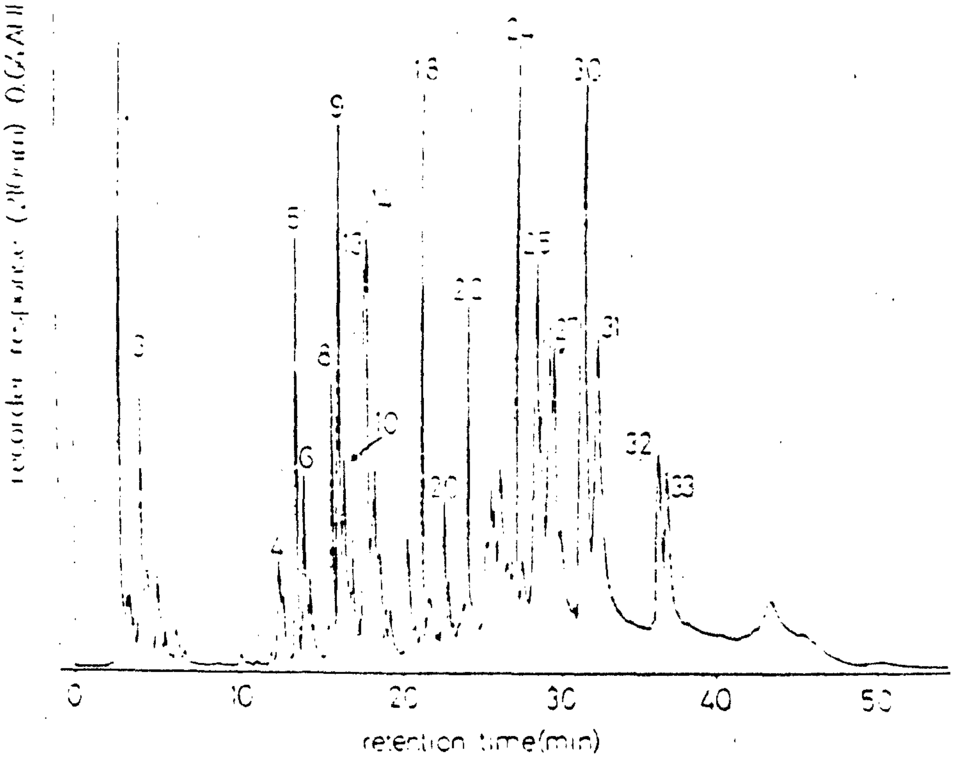


Fig. 1. Chromatogram of the venom reservoir extract.

References

1. Piek, T., Buitenhuis, A., Simonthomas, R.T., Ufkes, J.G.R. and Mantel, P. Comp.Biochem.Physiol. 1983, 75C, 145-152.
2. Piek, T., Mantel, P. and van Ginkel, C.J.W. Comp. Biochem. Physiol. 1984 78C, 473-474.
3. Piek, T., Hue, B., Mony, L., Nakajima, T., Pelhate, M. and Yasuhara, T. Comp. Biochem. Physiol. 1987, 87C, 287-295.

IVERMECTIN-STIMULATED RELEASE OF NEUROTRANSMITTER IN THE INSECT CENTRAL NERVOUS SYSTEM: MODULATION BY EXTERNAL CHLORIDE AND INHIBITION BY A NOVEL TRIOXABICYCLOOCTANE AND TWO POLYCHLOROCYCLOALKANE INSECTICIDES.

Russell A. Nicholson, Philip S. Robinson, Christopher J. Palmer* and John E. Casida*

Wellcome Research Laboratories, Berkhamsted, Hertfordshire, U.K. and *Pesticide Chemistry and Toxicology Laboratory, Department of Entomological Sciences. University of California, Berkeley, California, 94720, U.S.A.

The release of neurotransmitter from nerve terminals can be activated by a number of depolarising stimuli. Particular mechanisms include stimulation of sodium influx by sodium channel activators[1,2] or sodium ionophores[3], stimulation of calcium entry by neuroprotein agonists[4] or calcium ionophores[5] and elevation of extrasynaptosomal potassium concentration[6]. A recent investigation using insect synaptosomes in superfusion has indicated that the release of neurotransmitter induced by ivermectin may result from perturbation of the chloride ion gradient across the surface membrane of the nerve terminal[7]. Thus, neurotransmitter release is stimulated by ivermectin at low concentrations (EC_{50} 10^{-8}M) and in a manner that is inhibited by picrotoxinin [a specific blocker of the chloride channel[8] (Fig.1)] but not by tetrodotoxin [a selective sodium channel blocker[9] (no effect at 10^{-6}M)]. The present study uses trioxabicyclooctane and polychlorocycloalkane insecticides as modulators to further clarify the mechanism of action of ivermectin on insect nerve terminals.

Crude synaptosomal fractions were prepared from the central nervous system of the cockroach (Periplaneta americana), loaded with [^{3}H]-choline and superfused according to procedures already described[7]. Standard superfusion saline consisted of NaCl 215mM; KCl 3.1mM; $CaCl_2$ 2.04mM; Na_2HPO_4 3.29mM; NaH_2PO_4 0.19mM; $MgCl_2$ 6.6mM and glucose 16.5mM. Reductions to chloride ion concentrations in the superfusing saline were achieved by replacing sodium chloride with sodium acetate. Chloride-free medium was prepared by substituting calcium propionate for calcium chloride and potassium and magnesium sulphate for their respective chloride salts. High chloride superfusion medium was prepared by adding choline chloride to standard superfusion saline.

The efflux of radiolabel from synaptosomes (as a measure of neurotransmitter [^{3}H]-ACh release) is increased by ivermectin (10^{-8}M) under normal conditions of superfusion (115mM Cl^-) as noted previously[7]. Ivermectin-induced release is suppressed by 60% when extrasynaptosomal chloride is raised to 145mM and enhanced at chloride concentrations below 115mM. In the absence of chloride, ivermectin's stimulatory effect is increased by 68% compared to that in 115mM chloride medium.

The novel 4-t-butyl-3-cyano-1-(4-ethynylphenyl)-2,6,7-trioxabicyclo[2.2.2]octane[10] belongs to a class of convulsants known to have picrotoxinin-like actions at insect nerve-muscle junctions[11] and it is a highly potent inhibitor of GABA-mediated chloride influx in mammalian brain vesicles[10]. This trioxabicyclooctane is also an extremely potent inhibitor of ivermectin-induced transmitter release from cockroach synaptosomes (IC_{50}~2×10^{-9}M; Fig.1).

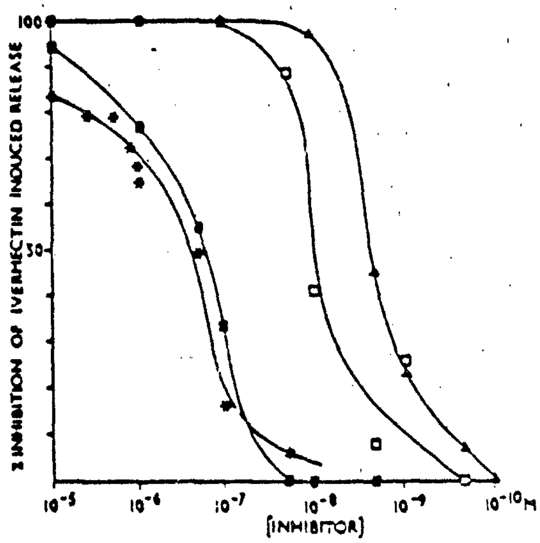


Figure 1. Inhibition of ivermectin-stimulated release of transmitter from synaptosomes by 4-t-butyl-3-cyano-1-(4-ethynylphenyl)-2,6,7-trioxabicyclo[2.2.2]octane (▲), endosulphan (□), bromocyclen (■) and picrotoxinin (✱).

In comparing with the polychlorocycloalkanes, endosulphan is a slightly weaker inhibitor and bromocyclen is similar in potency to picrotoxinin (Fig.1). Our previous study[7] establishes that γ-hexachlorocyclohexane antagonises the transmitter releasing action of ivermectin (IC_{50} $5 \times 10^{-8}M$). This action is clearly stereoselective as the β-isomer of hexachlorocyclohexane, which has no agonist activity alone, potentiates the ivermectin response. These findings with hexachlorocyclohexane isomers demonstrate that subtle structural changes can dramatically alter pharmacological properties.

In conclusion, the most likely mechanism of action of ivermectin in the insect central nervous system involves opening the chloride-specific ion channels in the surface membrane of the nerve terminal, thereby allowing an efflux of intraterminal chloride which in turn depolarises the nerve terminal and activates the secretory process. Similar anion-induced depolarisation effects are reported in dendritic regions of spinal cord neurones[12] and mammalian synaptosomal preparations[13].

1. Levi, G., Gallo, V. and Raiteri, M. Neurochem. Res. 1980, 5 281-295.

2. Nicholson, R.A., Wilson, R.G., Potter, C. and Black, M.H. Proc. 5th IUPAC. Int. Cong. of Pest. Chem., Kyoto, 1982 75-78.

3. Atwood, H.L., Charlton, M.P. and Thompson, C.S. J. Physiol. 1983, 335 179-195.

4. Crosland, R.D., Hsiao, T.H. and McClure, W.O. Biochemistry 1984, 23 734-741.

5. Redburn, D.A., Shelton, D. and Cotman, C.W. J. Neurochem. 1976, 26 297-303.

6. Askentsev, S.L., Rakovich, A.A., Okoon, I.M., Kouev, S.V., Orlov, S.N. and Kravtsov, G.M. Pflgers. Arch. 1983, 397 135-140.

7. Nicholson, R.A., Baines, P. and Robinson, P.S. Amer. Chem. Soc. Symp. Ser. 1987, No. 356 262-272.

8. Takeuchi, A. and Takeuchi, N. J. Gen. Physiol. 1969, 205 377-391.

9. Narahashi, T., Moore, J.W. and Scott, W.R. J. Gen. Physiol. 1964, 47 965-975.

10. Palmer, C.J. and Casida, J.E. unpublished results.

11. Casida, J.E., Palmer, C.J. and Cole. L.M. Mol. Pharmacol. 1985, 28 246-253.

12. Barker, J.L., Ransom, B.R. J. Physiol. 1978, 280 331-354.

13. Turner, J.D., Boakes, R.J., Hardy, J.A. and Virmani, M.A. J. Neurochem. 1987, 48 1060-1068.

PHYSICOCHEMICAL PROPERTIES OF PYRETHROIDS AND SODIUM CURRENTS IN CRAYFISH GIANT AXON.

Keiichiro Nishimura, Takamitsu Kobayashi and Toshio Fujita.

Department of Agricultural Chemistry, Kyoto University, Kyoto 606, Japan

We examined the effects of a set of pyrethroids, including variously substituted benzyl chrysanthemates and pyrethrates and their related compounds, on sodium currents in internally perfused giant axons of crayfish under voltage clamp conditions with a double sucrose-gap technique. The compounds increased the steady-state sodium current to induce a residual current during step depolarization of the axonal membrane. They also induced a tail current upon step depolarization.

The residual and tail currents increased with time after the start of the internal application of the compounds. The ratios of the residual and tail currents to the peak sodium current with step depolarization of the membrane at each measurement increased until a steady level was reached. The rate constants of the development of the residual and tail currents (k_R and k_T, respectively) in terms of the ratios were estimated by use of first-order kinetics. The rate constants were found to be independent of the pulse-duration and -amplitude and of the concentration of the compounds. Variations in the two kinds of log k values were analyzed with physicochemical parameters of the compounds by regression analysis with least-squares. The results showed that there was an optimum hydrophobicity for the rate of development of the residual and tail currents. The parabolic relationships with hydrophobicity probably

indicate that the development of the residual and tail currents is governed by non-specific penetration of the chemicals to the target site.

The tail current induced by the pyrethroids decayed exponentially with a time constant, τ. The τ value varied with the structure, but did not depend on the concentration of the compounds. Variations in the log τ value of the meta-substituted benzyl chrysanthemates, including phenothrin, were analyzed with the physicochemical parameters of the aromatic substituents. The results indicated that there is an optimum steric bulkiness in terms of the van der Waals volume[1] of the substituents. The shorter the substituent,[2] the greater is the log τ value. The compounds with substituents carrying an aromatic ring on the benzyl moiety had activity enhanced by about 30 times over that of compounds with non-aromatic substituents. The time constant of pyrethroid-modified sodium channels to close upon repolarization of the membrane was mostly governed by steric dimensions and the aromaticity of substituents on the benzyl moiety of the chrysanthemates.

1. Bondi, A. J. Phys. Chem. 1964 68 441-451
2. Verloop, A., Hoogenstraaten, W. and Tipker, J. Drug Design, Vol. 7, Academic Press, New York, 1976, pp. 165-207

A Comparison of the Effects of Venoms from Four Spider Families on Neuromuscular Synapses of Insects.

David R. Pepper and Ian R. Duce

Department of Zoology, University of Nottingham, University Park, Nottingham NG7 2RD, England.

A single species of British spider was selected to represent each of four different families. Families were selected on the basis of different prey and predation strategies. Species were chosen for their availability, size and likely venom yield, and were as follows:

Family	Species	Prey-Capture
Araneidae	*Araneus diadematus*	Orb-web.
Theridiidae	*Steatoda bipunctata*	3 dimensional web.
Agelenidae	*Tegenaria gigantea*	Horizontal sheet web.
Lycosidae	*Pardosa amentata*	No web, active hunter.

Araneus diadematus and *Tegenaria gigantea* venoms were collected using an electrical milking technique. The yields of venom were 0.32µl for *A diadematus* and 0.4µl for *T. gigantea*. Venom from the other species was extracted by freezing and partially thawing the cephalothorax, pulling out the venom glands and chelicerae and homogenising them in distilled water. The homogenate was centrifuged twice at 15,000 x g and the supernatant (referred to as venom gland extract - VGE) was tested for activity. All venoms and VGE were stored at -80°C.

Milked venom and VGE activity was initially tested by injecting *Calliphora* larvae with various concentrations and examining the dose required to produce paralysis. Venoms from *A. diadematus* and *T. gigantea* induced flaccid paralysis with ED_{50} of 0.08µl and 0.025µl respectively whereas *S. bipunctata* venom produced tetanic paralysis with an ED_{50} of 0.014 µl VGE (yield of VGE/spider 2.88µl). Insufficient venom was collected from *P. amentata* to obtain meaningful dose response data.

The effects of venom on the neurally evoked twitch contraction of the locust retractor unguis muscle were monitored as previously described[1]. *A. diadematus* venom produced an activation induced inhibition of muscle contraction as reported for other araneid venoms[1]. *T. gigantea* venom produced a qualitatively similar toxicity (Fig.1), consistent with the flaccid paralysis of intact larvae. The use dependent nature of this inhibition suggests that at least one component of *Tegenaria* venom is acting postsynaptically, possibly at the level of the glutamate-gated ion channel. The effects of both *A.diadematus* and *T. gigantea* venoms were reversible after extensive washing. *S. bipunctata* venom on the other hand produced a very different toxicity (Fig. 2). An

initial reduction in twitch amplitude was followed by a large contracture of the muscle which was irreversible, following washing for one hour. Examination of the ultrastructure of treated muscles 5 minutes and 60 minutes after venom application revealed changes in morphology. After five minutes some nerve terminals were depleted of vesicles and mitochondria were swollen. After 60 minutes nerve terminals were totally disrupted and the muscle was extensively damaged. These observations imply that S.bipunctata venom acts initially at the pre-synaptic element of the neuromuscular junction, to induce the release of neurotransmitter. Subsequent damage to the muscle may be secondary or may result from other venom components. P.amentata venom produced an inhibition of the muscle twitch which could not be reversed by washing. However this venom produced marked muscle damage which may be reponsible for the inhibition of contraction.

D.P. is a NERC research student

1. Bateman, A. Boden, P. Dell, A. Duce, I.R. Quicke, D.L.J. and Usherwood, P.N.R. Brain Res. 339 237-244 (1985).

Figs. 1 and 2 The effects of spider venoms on the neurally evoked twitch contraction of the locust retractor unguis muscle. Upward deflections represent evoked contractions: stimulation frequency 0.3Hz. Arrows signify addition or removal of venom.

Fig.1 0.2µl T.gigantea milked venom

Fig.2 1.0µl S.bipunctata VGE

AMPULEX COMPRESSA CHANGES THE BEHAVIOUR OF THE COCKROACH

Tom Piek[1], Bernard Hue[2], Albert Lind[1], Piet Mantel[1], Jan van Marle[3] and Hans H. Visser[4]

Departments of Pharmacology[1], Electronmicroscopy[3], University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands, Department of Neurophysiology[2], UA CNRS no 611, University of Angers, 49045 Angers Cedex, France and Department of Entomology[4], Agricultural University, Wageningen, The Netherlands.

The solitary wasp Ampulex compressa stings cockroaches, Periplaneta americana, two times. The first sting in the ventral thorax results in a transient paralysis. During that period of paralysis the wasp stings in the suboesophageal ganglion, which results in a delayed and permanent deactivation[1]. The ductus venatus shows a large lumen with a folded cuticula. No venom reservoir is present. An extract of the venom gland causes a contraction of the guinea pig ileum with a slow time course. The agonist cannot be identified as a known agonist. The venom gland extract blocks synaptic transmission from the cercal nerve to giant neurons in the sixth abdominal ganglion of the cockroach with a delay, which is comparable to that of the deactivation.

References

1. Piek, T., Visser, J.H. and Veenendaal, R.L. Entomol.exp.appl. 1984 35, 195-203.

EFFECTS OF THE VENOM OF COLPA INTERRUPTA ON VERTEBRATE SMOOTH MUSCLE AND SYNAPTIC TRANSMISSION IN INSECT CNS

Tom Piek[1], Bernard Hue[2], Piet Mantel[1], Terumi Nakajima[3], Marcel Pelhate[2] and Tadashi Yasuhara[3].

[1]Department of Pharmacology, Univ. of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, [2]Department of Neurophysiology, UA CNRS no 611, Univ.of Angers. 49045 Angers Cedex, France and [3]Faculty of Pharmaceutical Sciences, Bunkyo-ku Tokyo 113, Japan.

Females of *Colpa interrupta* (F.) (= *Campsomeris sexmaculata* (F.)) were collected in the south of France and killed and transported in liquid nitrogen. The venom reservoirs were removed from freshly thawed gasters and extracted in distilled water. The freeze-dried venom was chromatographed three times using a LS-80, TmODS column and eluted with a gradient from 5% CH_3CN, 0.05% TFA to 60% CH_3CH, 0.05% TFA. The combined 15 fractions were tested pharmacologically using a cascade of vertebrate smooth muscle preparations[1]. The venom and fraction 9 contain a kinin-like activity and a second agonist which causes contractions of the muscle preparations, including the rat duodenum and colon, which with a kinin show relaxation, and inhibition of spontaneous activity respectively.

Similar to the venom[2], fraction 9 has no effect on the excitability of the giant axon of the cockroach, but blocks excitatory synaptic transmission from the cercal nerve to the giant interneuron in the sixth abdominal ganglion, with a delay.

References

1. Piek, T. and Spanjer, W. In: Venoms of the Hymenoptera (T. Piek, editor) p.218-219. Academic Press, London, 1986.
2. Piek, T., Hue, B., Pelhate, M. and Mony, L. Comp. Biochem. Physiol. 1987, 87C, 283-286

DESTRUXINS, FUNGAL TOXINS THAT ACTIVATE CALCIUM CHANNELS IN INSECT MUSCLE.

Stuart E. Reynolds, Richard I. Samuels and A. Keith Charnley

School of Biological Sciences, University of Bath, Claverton Down, Bath, England, BA2 7AY.

Destruxins are a family of cyclodepsipeptide toxins produced by the entomopathogenic fungus Metarhizium anisopliae. The toxins are produced in culture and also during the course of fungal infections of insects[1]. Destruxins cause paralysis by depolarising the insect's muscles, thus causing them to contract[2].

Pharmacological and ion-substitution experiments suggest that destruxins act on the muscle membrane by making the cell permeable to Ca^{2+} ions. Thus, destruxin-induced depolarisation is blocked by cadmium ions, and by nifedipine, both well-known Ca channel blockers, and by removing divalent cations from the bathing medium.

The way in which destruxins act to increase Ca^{2+} permeability is not certain. Although the structure of the destruxin molecule superficially resembles that of an ionophore, experiments with Pressman Cells and resealed red cell ghosts have failed to yield evidence of ionophoric activity. Thus we suggest that destruxins act by directly opening endogenous Ca channels.

References

1. Samuels, R.I., Reynolds, S.E. and Charnley, A.K. Pestic.Sci. 1985, 16 450–451.

2. Samuels, R.I., Charnley, A.K. and Reynolds, S.E. J.Chromatogr.Sci. 1988, 26, 15–19.

DISTRIBUTION OF FMRFamide-LIKE PEPTIDES IN THE LOCUST NERVOUS SYSTEM.

Sandra Robb and Peter D. Evans.

AFRC Unit, Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ.

The tetrapeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) was first isolated from the central nervous system of the clam, Macrocallista nimbosa[1,2]. Subsequently, FMRFamide and other structurally related FMRFamide-like compounds have been identified in many invertebrate and vertebrate species[3]. Recently FMRFamide-like peptides have been identified immunocytochemically in the nervous system of the locust, Schistocerca gregaria[4,5,6]. In addition FMRFamide and a range of structurally related analogues have been shown to modulate neuromuscular transmission and muscle contraction in locust skeletal muscle[7,8] and to modulate the contractile properties of the locust heart[9,10].

In the present study we have used radioimmunoassay (RIA) techniques to study the distribution of FMRFamide-like peptides in the locust nervous system and various peripheral tissues, and combined the technique with HPLC procedures to partially characterise the FMRFamide-like immunoreactivity present in different regions of the locust nervous system.

Locust tissues were extracted in acidified methanol (90% methanol, 9% acetic acid, 1% distilled water) and their FMRFamide-like peptide content assessed by RIA after using synthetic FMRFamide to construct dose-response curves. All the results are calculated on the basis of FMRFamide equivalents. The results show that the levels of FMRFamide-like immunoreactivity vary widely in different parts of the nervous system of the locust. Relatively large amounts of immunoreactive material are contained in the brain and thoracic ganglia. Other tissues, such as the abdominal nerve cord and terminal abdominal ganglion contain relatively small amounts. The distribution of FMRFamide-like immunoreactivity

measured using RIA is in good agreement with previous immunocytochemical findings.

The chemical nature of the endogenous FMRFamide-like peptides in the locust has been investigated using HPLC t· hniques. Crude extracts of locust tissues were initially applied to G15 Sephadex gel filtration column. FMRFamide-like immunoreactive material was eluted with 0.1M acetic acid and active fractions pooled and concentrated in a Speed Vac vaccuum centrifuge. Samples were cleaned by passage through C_{18} Sep Paks prior to application to the HPLC column. The FMRFamide-like immunoreactivity was characterised from each of the different tissue extracts by using three to four different solvent systems run on a reversed phase C_{18} μ Bondapak (Waters) column. HPLC fractions were anilysed by RIA for their FMRFamide-like immunoreactivity and were also bioassayed for FMRFamide-like activity on both the locust extensor-tibiae muscle and the locust heart.

The chromatographic results suggest that the different locust tissues examined contain at least 2-6 different FMRFamide-like peptides which confirms the findings from immunocytochemical techniques. The results suggest that little or no authentic FMRFa·ide-like is present in the locust nervous system but that the endogenous FMRFamide-like material probably consists of N-terminally extended peptides. The complement of FMRFamide-like peptides differs in each of the tissues examined. The amino acid sequences of the endogenous FMRFamide-like peptides in the locust nervous system are currently under investigation.

1. Price, D.A. and Greenberg, M.J. (1977) Science, 197,670-671.
2. Price, D.A. and Greenberg, M.J. (1977) Prep. Biochem. 7, 261-281.
3. O'Shea, M. and Schaffer, M. (1985) Ann. Rev. Neurosci. 8, 171-198.
4. Myers, C.M. and Evans, P.D. (1985) J. Comp. Neurol. 234, 1-16.
5. Myers, C.M. and Evans, P.D. (1985) Cell and Tissue Res. 242 109-114..
6. Myers, C.M. and Evans, P.D. (1987) Cell and Tissue Res. 250, 93-99.
7. Walther, C., Schiebe, M. and Voigt, K.H. (1984) Neurosci. Letters. 45, 99-104.
8. Evans, P.D. and Myers, C.M. (1986) J. exp. Biol. 126, 403-422.
9. Myers, C.M. (1986) Ph.D. Thesis, University of Cambridge.
10. Cuthbert, B.A. and Evans. P.D. (1988) in preparation.

AVERMECTIN BINDING IN CAENORHABDITIS ELEGANS: A TWO-STATE MODEL FOR THE AVERMECTIN BINDING SITE

James M. Schaeffer, Herbert W. Haines and Mervyn J. Turner

Merck Sharp & Dohme Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065, U.S.A.

Avermectins (AVM) are a family of macrocyclic lactones isolated from Streptomyces avermitilis which have potent anthelmintic and insecticidal activity. Structurally, AVMs possess a 16-membered lactone ring with a spiroacetal system consisting of two six-membered rings and an α-L-oleandrosyl-α-L-oleandrosyloxy disaccharide substituent at the C-13 position. The naturally occurring AVMs can be separated into four major components (A_{1a}, A_{2a}, B_{1a} and B_{2a}), of which the B series are generally more biologically active. A synthetic derivative of B_1, 22-23-dihydroavermectin (B_1 ivermectin, IVM) has been developed for veterinary and medical use.

Specific high affinity [^{3}H] IVM binding sites have been identified in rat and dog brain preparations[1,2], however, no studies have described the presence of specific AVM binding sites in tissues from AVM-sensitive target organisms. In this report, we describe the binding of [^{3}H] IVM to membranes isolated from Caenorhabditis elegans, a free living nematode which is extremely sensitive to AVM. The characteristics of [^{3}H] IVM binding to C. elegans membranes are distinct from binding in mammalian systems, and may enable us to further understand the mechanism by which this class of anthelmintic compounds works.

Specific [^{3}H] IVM binding to C. elegans membranes is saturable with an apparent dissociation constant, K_D, of 0.26 nM and a receptor concentration of 3.53 pmol/mg protein. [^{3}H] IVM binding in C. elegans is linear with tissue protein concentration and optimal binding occurs within a pH range of 7.3 to 7.6. Kinetic analysis of the binding showed that the reaction proceeds by a two-step mechanism. Initially, a rapidly reversible complex is formed and after additional incubation this complex is transformed to a much more slowly reversible complex. Stereospecificity of [^{3}H] IVM binding to C. elegans membranes was demonstrated by competition with a series of AVM derivatives. The in vivo effect of IVM and its derivatives on C. elegans motility is concentration dependent and correlates well with their relative binding affinities. Several putative neurotransmitters including GABA, carbamyl choline, taurine, glutamate and dopamine were tested and found to have no effect on IVM binding. Specific IVM binding

sites were also identified in rat brain, however, the affinity is approximately 100-fold lower than that observed in _C. elegans_ and stereospecificity studies demonstrate significant structural differences in the two binding sites. These results are the first direct demonstration of a specific IVM binding site in nematodes and thus important in further understanding its mode of action.

1. Pong, S.S. and Wang, C.C. Neuropharmacol. 1980 19 311-317
2. Drexler, G. and Sieghart, W. Eur. J. Pharmacol. 1984 99 269-277

ARGIOPINE DIFFERENTIATES BETWEEN VERTEBRATE AND INVERTEBRATE GLUTAMATE BINDING SITES

James M. Schaeffer, Thomas L. Shih, Mervyn J. Turner and Helmut Mrozik

Merck Sharp & Dohme Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065, United States

Several spider venoms contain components which specifically inhibit the postsynaptic response of cells to glutamate stimulation. The chemical identities of several of these toxins have been determined. Argiopine was first isolated from Argiope lobata[1] and more recently, structurally similar toxins have been isolated from Argiope aurantia[2], Araneus gemma[3], Argiope trifasciata[3], Nephilia clavata[4] and Nephilia maculata[4]. It has been postulated that these toxins inhibit the EPSP by directly binding to the postsynaptic glutamate binding site or via a noncompetitive inhibition of the glutamate regulated ion channel, however, their precise mechanism of action remains unclear. Our laboratory has recently characterized a specific, high affinity glutamate binding site associated with membranes isolated from the nematode, Caenorhabditis elegans. In this poster we describe the effect of synthetic argiopine on glutamate binding to C. elegans membranes in order to characterize the interaction between argiopine and the glutamate receptor.

Argiopine inhibits specific [^{3}H] glutamate binding to membranes isolated from C. elegans with a dissociation constant, K_I, of 8μM. Conversely, argiopine has no inhibitory effect on specific glutamate binding to membranes isolated from rat brain, and at concentrations greater than 10 μM argiopine stimulates glutamate binding. To further characterize argiopine inhibition of [^{3}H] glutamate binding, C. elegans membranes were incubated with increasing concentrations of [^{3}H] glutamate in the presence or absence of a fixed concentration of argiopine (5, 10 and 15 μM). The double reciprocal plot of this data yields a series of lines intersecting at the y-axis, indicating that argiopine is a competitive inhibitor of glutamate binding to sites in C. elegans membranes with an inhibition constant of 6 μM. The affinity of glutamate binding to C. elegans membranes in the absence of argiopine is 0.42 μM.

In order to evaluate the in vivo nematocidal activity of argiopine, C. elegans were incubated in the presence of increasing concentrations of argiopine and the percentage of motile worms was quantitated after 24 hours. The ED_{50} (concentration at which 50% of the worms remain motile) is 25 μM. The addition of exogenous glutamate does not alter the argiopine effect.

These findings demonstrate that the nematode glutamate binding site is pharmacologically distinct from the mammalian site and represents a target for the development of compounds with specific toxicity to nematodes.

1. Grishin, E.V., Volkova, T.M., Arsenev, A.S., Reshetova, O.S., Onoprienko, V.V., Maganzanik, I.G., Antonov, S.M. and Fedorova, I.M. Bioorgan. Khim. 1986 12 1121-1124

2. Adams, M.E., Carney, R.L., Enderlin, F.E., Fu, E.T., Jarema, M.A., Li, J.P., Miller, C.A., Schooley, D.A., Shapiro, M.J. and Venema, V.J. Biochem. Biophys. Res. Comm. 1987 148 678-683.

3. Aramaki, Y., Yasuhara, T., Higashijima, T., Yoshioka, M., Miwa, A., Kawai, N. and Nakajima, T. Progr. Japan Acad. 1986 62 359-362

4. Hashimoto, Y., Endo, Y., Shudo, K., Aramaki, Y., Kawai, N. and Nakajima, T. Tetr. Letters 1987 28 3511-3514

INHIBITION OF TRANSMISSION AND REDUCTION OF GLUTAMATE UPTAKE IN RAT HIPPOCAMPUS BY DELTA-PHILANTHOTOXIN

Nico C.M. Schluter, Albert Lind, Jan van Marle* and Janneke van Weeren-Kramer

Department of Pharmacology and Electronmicroscopy*, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

The wasp Philanthus triangulum paralyses workers of the honeybee Apis mellifera by blocking the excitatory neuromuscular transmission. This glutamatergic transmission is affected by delta-philanthotoxin (δ-PTX) at two different synergistically acting processes, a presynaptic reduction of the high affinity glutamate reuptake[1] and a postsynaptic block of the glutamatergic cation channel[2,3]. We have studied both glutamate reuptake and the glutamatergic excitatory neurotransmission in rat hippocampal slices. For uptake experiments 200 μm slices were prepared and incubated under high affinity conditions. The preparations were incubated in 10 μM of δ-PTX for 30 min resulting in a reduction of 74% of the glutamate uptake. The effect on excitatory synaptic transmission was studied by recording field potentials from the stratum radiatum and stratum pyramidale of the CA1 region of a hippocampus slice (400μ) evoked by paired stimuli (interval 20 ms) given to the Schaffer collaterals. Substancial inhibition of the transmission occurred at 800 μM of the synthetic toxin δ-PTX-S_1, which probably contains 400 μM of the active isomer δ-PTX (Piek et al. this meeting). This has been found for both the excitatory postsynaptic potential and the population spike.

References

1. Van Marle, J., Piek, T., Lind, A. and Van Weeren-Kramer. J. Comp. Biochem.Physiol. 1984, 79C, 213-215.
2. Clark, R.B., Donaldson, P.L., Cration, K.A.F., Lambert, J.J., Piek, T. Ramsey, R., Spanjer, W. and Usherwood, P.N.R. Brain Res. 1982, 241, 105-114.
3. Piek, T. Comp. Biochem.Physiol. 1982, 72C, 311-315.

MOLECULAR PROPERTIES OF THE APAMIN BINDING PROTEIN ASSOCIATED WITH A CALCIUM ACTIVATED POTASSIUM CHANNEL

Michael J. Seagar, Beatrice Marqueze, and Francois Couraud.

Laboratoire de Biochimie, CNRS UA 1179- INSERM U172, Faculte de Medecine Nord, Bd. Pierre Dramard, 13326 Marseille Cedex 15, France.

Apamin, a 2kDa peptide from bee venom, is a potent convulsant in the mammalian CNS with a LD_{50} of about 10 ng/mouse by intracerebroventricular injection. Its neurotoxic action has been attributed to specific blockade, at nanomolar concentrations, of the slow Ca^{++} activated K^{+} current (I_{AHP}) that underlies the late afterhyperpolarization in certain neurons.[1,2]. It is however inactive on the rapid voltage sensitive Ca^{++} current (I_C) responsible for spike repolarization and early hyperpolarization which is probably carried by high conductance BK channels.[1,2].

Mono [^{125}I] iodoapamin binds with high affinity to intact cultured neurons (K_D= 90pM, B_{max}= 500-1000 sites/cell) and synaptic membranes (K_D= 30pM, B_{max}= 30fmol/mg protein)[3,4]. It is therefore a potentially useful K^{+} channel probe, although the very low receptor density is a limiting factor.

Apamin contains two primary amines (alpha cys_1 and epsilon lys_4) that can be modified to produce photoactivable arylazide derivatives. Photoaffinity labeling with these derivatives has identified receptor polypeptides of **86, 59 kDa** accessible from the alpha cys_1 position and **33 kDa** accessible from the epsilon lys_4 position of the bound ligand. This suggests that apamin binds at the interface between 3 putative K^{+} channel subunits[5]. Radiation inactivation studies were carried out to determine which of these polypeptides functionally constitute(s) the binding site. Increasing exposure of frozen brain membranes to 10MeV electrons lead to a monoexponential decrease in receptor capacity indicating a target size of **84-115 kDa**[5]. The simplest interpretation is that the 86 kDa chain alone carries the neurotoxin binding site.

The apamin binding protein can be extracted from rat brain membranes using sodium cholate. Scatchard analysis of equilibrium binding data revealed a single class of non-interacting sites with K_D=40pM and B_{max}=17fmol/mg. protein. As in membrane inserted receptors apamin binding is stimulated by occupation of a K^{+} ion site that saturates at low millimolar concentrations. Other cations can be substituted for K^{+} with an affinity sequence: K^{+}=Tl^{+}=Rb^{+}> Cs^{+}> NH_4^{+}> Li^{+} or Na^{+}. This K^{+} selective site may be associated with the ion permeation pathway implying that the solubilized receptor is closely linked to the K^{+} channel. The traditional K^{+} channel blockers quinidine and tetraethylammonium^{+} displaced apamin

from its solubilized receptor at concentrations similar to those required to block flux through apamin sensitive channels[6].

The apamin binding protein extracted with sodium cholate thus retains certain properties indicating that the K^+ channel is part of the same solubilized protein. Analysis by sucrose gradient centrifugation showed that the receptor sediments with $S_{20,w} = 20$, corresponding to a molecular weight of about 700kDa, however as much as 50% of this could be contributed by the detergent[6]

Receptor sites for ^{125}I-apamin have also been detected and identified by photoaffinity labeling in primary cultured astrocytes and heart cells as well as in membrane preparations from liver and smooth muscle. A **86-87kDa** component was present in all these tissues whereas the **57-59 and 33kDa** polypeptides were revealed in some but not in others. These observations support the hypothesis that the larger chain contains the apamin binding site while the two smaller polypeptides are noncovalently associated peripheral subunits[7,8].

The fact that glial cells possess apamin receptors and presumably therefore apamin sensitive K^+ channels is of particular interest. Astrocytes are known to be involved in redistributing K^+ in the central nervous system. If these channels play a role in this process their inhibition could increase the extracellular K^+ concentration which would stimulate neuronal firing and contribute to apamins central neurotoxicity[7]

References.

1. Romey,G., and Lazdunski,M. (1984) Biochem. Biophys. Res. Comm. 118, 669-674
2. Pennefather,P., Lancaster,B., Adams,P.R. and Nicoll,R.A. (1985) Proc. Natl. Acad. Sci. (USA) 82, 3040-3044
3. Hugues,M., Duval,D., Kitabgi,P., Lazdunski,M., and Vincent,J-P. (1982) J. Biol. Chem. 257, 2762-2769
4. Seagar,M.J., Granier,C. and Couraud,F. (1984) J. Biol. Chem. 259,1491-1495
5. Seagar,M.J., Labbe-Jullie,C., Granier,C., Goll,A., Glossmann, H.,Van Rietschoten,J., and Couraud,F. (1986) Biochemistry. 25. 4051-4057
6. Seagar,M.J., Marqueze,B., and Couraud,F. (1987) J. Neurosci. 7. 565-570
7. Seagar,M.J., Deprez,P., Martin-Moutot,N. and Couraud,F. (1987) Brain Res. 411, 226-230
8. Marqueze,B., Seagar,M.J., and Couraud,F. (in press) Eur. J. Biochem.

REVERSAL OF BOTULINUM TOXIN SYNAPTIC BLOCKADE BY NICOTINAMIDE AT THE RAT NEUROMUSCULAR JUNCTION.

R.A. Shiells and G. Falk,
Biophysics Unit, Physiology Department, University College,
London, Gower Street, London, WC1E 6BT.

There is increasing evidence indicating that botulinum toxin type D inhibits Ca^{2+} - dependent exocytosis via ADP-ribosylation of intracellular proteins[1,2]. The question arises whether this is the mechanism by which the toxin interrupts synaptic transmission at the neuromuscular junction. We have demonstrated that nicotinamide, a known inhibitor of ADP-ribosylation, reverses the effect of pertussis toxin which ADP-ribosylates the G-protein in retinal rods[3,4].

Rat diaphragm nerve-muscle preparations were incubated with botulinum toxin type D (2.5 μg/ml) in the presence or absence of nicotinamide (10 mM). Contractions persisted in the presence of nicotinamide up to 1 hr after addition of the toxin. To ensure toxin entry into nerve terminals, incubations were made for 4 hrs in oxygenated L-15 culture medium (Sigma) at 35°C. Intracellular recordings of miniature end-plate potentials (m.e.p.p.s) were obtained from end plate regions of muscle fibres after incubation with nicotinamide and toxin (Fig. 1.a.). Removal of nicotinamide resulted in a progressive reduction in m.e.p.p. amplitude and frequency (b,c.) over a period of 20 minutes indicating toxin action. Subsequent re-application of nicotinamide to the bathing medium resulted in a progressive recovery of m.e.p.p. amplitude and frequency over a similar time course (d,e.). Blockade of the spontaneous

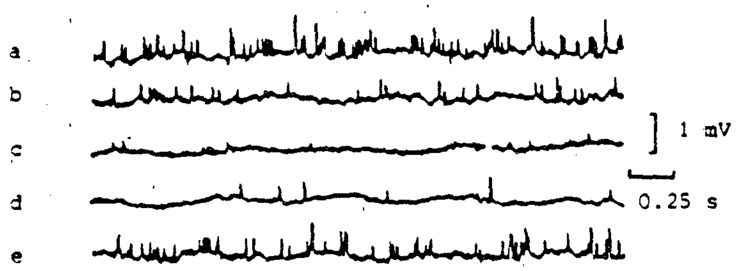


Fig. 1. Reversal of botulinum toxin action by nicotinamide. The membrane potential decreased from -67 mV in (a) to -55 mV in (e) during the recording.

release of transmitter from nerve terminals may be used as an indication of neuromuscular transmission blockade by botulinum toxin[5]. While the results provide strong evidence that botulinum toxin type D interrupts neuromuscular transmission by ADP-ribsylation of intracellular proteins, the definitive experiments on evoked end-plate responses have yet to be done. Nicotinamide and cogeners may be effective in the treatment of botulism, and other diseases caused by ADP-ribosylating toxin-producing pathogens

We would like to thank D.E. Knight for assistance and advice and the M.R.C. for a project grant.

REFERENCES

1. Ohashi, Y & Naramiya, S. 1987. J. Biol. Chem. 262, 1430-1433.
2. Adam-Vizi, V. & Knight, D.E. 1987. J.Physiol. 394, 96P.
3. Falk, G. & Shiells, R.A. 1987. J. Physiol. 1988. 396 125P.
4. Falk, G. & Shiells, R.A. FEBS Letts. (in press)
5. Cull-Candy, S.g., Lundh, H. & Thesleff, S. 1976. J. Physiol. 260, 177-204.

EFFECTS OF IVERMECTIN AND RELATED COMPOUNDS ON STRETCHER MUSCLE OF THE LINED SHORE CRAB (PACHYGRAPSUS CRASSIPES): ELECTROPHYSIOLOGICAL RESPONSES AND CORRELATIONS WITH ANTHELMINTIC ACTIVITES.

David P. Thompson, Lamar Lee and Jerry W. Bowman

Parasitology Research, The Upjohn Company, Kalamazoo, MI, USA 49001

Effects of Ivermectin (IVM) and several related compounds on membrane electrical properties of shore crab stretcher muscle were examined using standard intracellular techniques. Results of these studies show IVM reduces input resistance (Rm) while slightly hyperpolarizing muscle fibers in a concentration-dependent manner, beginning at 10 nM. At 1 µM, IVM reduces Rm by 90% within 1 min, and by 85% within 15 min. Replacement of IVM with Cl^- channel blockers (picrotoxinin or lindane) results in partial recovery of Rm. Channel blockers, specific for other ions, including TEA, 4-AP, TTX and D-600 do not antagonize resistance changes due to IVM. Muscle fibers bathed in medium containing 20 mM Co^{2+}, 0.1 mM bicuculline, or predesensitized with 1 mM GABA remain highly responsive to IVM, suggesting the compound's actions on this preparation are postsynaptic, yet not dependent on stimulation of the GABA receptor site *per se*. Results of similar tests using analogs of IVM reveal that relatively minor structural changes often result in profound loss of biological activity. In general, analogs that cause conductance increases in shore crab muscle also exhibit anthelmintic activity at similar concentrations against the free-living nematode, *Caenorhabditis elegans*. When exceptions occur within the range of therapeutic levels, as with the C-5 ketone or 6,28-deoxy derivatives of IVM, weaker activity in the anthelmintic assay may be predicted on the basis of picrotoxinin irreversibility in the shore crab muscle assay. That is, unlike other analogs tested, only C-5 ketone and 6,28deoxy derivatives of IVM stimulate a conductance which is not antagonized by picrotoxinin or lindane.

SYNTHESIS OF Δ-PHILANTHOTOXIN

Yulan C. Tong

Agricultural Products Department, Dow Chemical USA
P.O. Box 9002, Walnut Creek, Ca. 94598, U.S.A.

One of the components of the venom of the solitary digger wasp, *Philanthus triangulum*, was designated δ-philanthotoxin. Its structure was studied by Piek et al.[1] We synthesized a racemic sample, which was found to be chemically identical to the natural product. Biologically, the synthetic sample was half as active.

The synthetic scheme will be presented.

1. Piek, T., Private communication. Structure to be presented at *NEUROTOX'88*, Nottingham, England.

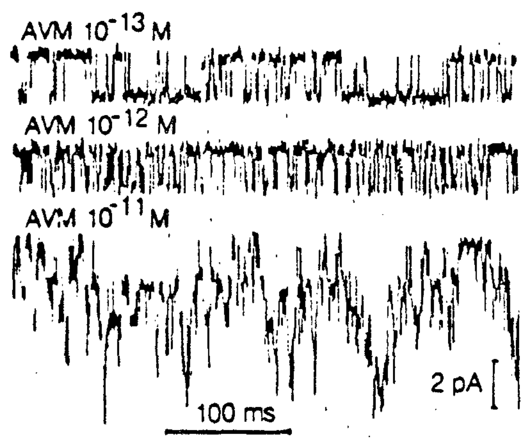
AVERMECTIN B_{1A} DIRECTLY OPENS THE MULTI-TRANSMITTER GATED CHLORIDE CHANNEL IN CRAYFISH MUSCLE.

Frank Zufall, Christian Franke and Hanns Hatt

Physiologisches Institut der TUM, Biedersteiner Strasse 29, 8000 München 40, F.R.G.

We have recently shown with the patch-clamp technique, that glutamate, GABA and acetylcholine (Ach) open the same chloride channel in opener and stomach muscle of the crayfish[1,2]. Here we present some results of the action of the insecticide avermectin B_{1A} (AVM) on these chloride channels.

Superfusion of excised outside-out patches of the crayfish muscle membrane with low doses of AVM (10^{-13}-10^{-12} M) leads to a reversible opening of chloride channels with the same conductance (22 pS) as those activated by glutamate and Ach,



whereas GABA mainly activates an open state with a doubled conductance level. AVM does not activate the excitatory glutamate-activated cation channel, which is present on these patches too.

The distribution of lifetimes of openings elicited by AVM could be fitted by a single exponential with a time

constant of about 2,5 -3 ms corresponding to the mean open time, which lies in the same range as those produced by Ach and glutamate, but is strongly reduced by increasing the concentration of the insecticide. Reducing the extra-cellular Ca^{2+}-concentration induces the same effect in activation and kinetics as raising the AVM-concentration. For the AVM-induced activation no desensitization is apparent. The bursting behaviour of these channels, which is normally seen by activation with glutamate, Ach or GABA, is strongly reduced with AVM.

On inside-out patches, AVM applied to the inner side of the membrane, does not open any channel. This finding leads to the conclusion, that AVM does not act from the inside and is not able to cross the membrane.

If the AVM concentration is raised above 10^{-11} M, an enormous increase in the rate of openings of the 22 pS channel within several seconds occurs, which cannot be washed out during the lifetime of the patch (30-60 min). High doses of picrotoxin (10^{-3} M) can reduce this activity up to 50 %. The high channel opening rate is fully restored in physiological solution after the picrotoxin is taken out. However, if picrotoxin is applied first, the irreversible action of AVM is completely abolished.

These findings support the idea of an irreversible binding of AVM to the chloride channel receptor complex.

1. Franke, Ch., Hatt, H. and Dudel, J. J. Comp. Physiol. A 1986 159 591-609
2. Zufall, F., Franke, Ch. and Hatt, H. J. Comp. Physiol. A 1988 in press

B. PHYSIOLOGICAL ASPECTS OF RECEPTORS AND ION CHANNELS

GIGA-OHM RECORDINGS OF GLUTAMATE-GATED CHANNELS

FROM ADULT LOCUST MUSCLE

Susan E Bates, Robert L Ramsey and Peter N R Usherwood

Department of Zoology, The University of Nottingham, Nottingham, England NG7 2RD

Although extensive investigations of L-glutamate-gated D-receptor channels[1] in adult locust muscle have been carried out, using the mega-ohm seal technique[2,3], studies of these channels under giga-ohm seal conditions have, until recently[4,5], been confined to cultured, embryonic muscle[6]. This appears to be due to the presence, in the adult, of an extensive glycocalyx, covering the surface of the muscle membrane, which prevents the formation of high resistance seals between patch pipette and muscle membrane. In the present study a cocktail of enzymes - of which collagenase is the main constituent - has been used to remove sufficient of the glycocalyx to allow giga-ohm seals to be formed.

The extensor tibiae muscle of the metathoracic leg of the desert locust, Schistocerca gregaria was treated with collagenase (sigma, type 1A) at a concentration of 2 mg/ml for 1½ to 2 hours at a temperature of 27-28° C. After this period, giga-ohm seals can be formed readily, using either polished or unpolished patch pipettes. In order to observe non-desensitising ion channels in the presence of L-glutamate, it is necessary to apply the Lectin Concanavalin A to the muscle[7]. The application of Con A to collagenase-treated muscle causes extensive damage to the muscle fibres, and it is difficult to form giga-ohm seals on preparations which have been exposed to both of these treatments. To avoid these problems, Con A was a applied to outside-out patches excised from collagenase-treated muscle.

Patch pipettes contained locust saline with a low chloride ion concentration (19 mM) in order to adjust the reversal potential of the hyperpolarising glutamate-gated chloride channels[8], large numbers of which are observed in most patches. Also, the pipette saline contained zero Ca^{2+} and, in some experiments K^+ was also omitted, in order to minimise the influence of K^+ channels.

Outside-out patches were first exposed to a conditioning pulse of L-glu in order to establish whether glu receptors were present. Approximately 50% of patches respond to such pulses. If channels were present, the patches were then continuously perfused with Con A for up to 6 minutes. After this period, non-desensitising glu channels could be recorded from most of the patches.

The single channel conductance of the depolarising channel was similar to that seen with the mega-ohm technique ie about 120 pS. Concentrations of glu between 10^{-4} M and 10^{-2} M have been investigated. At the higher glutamate concentrations, more than one channel was usually seen after Con A application although, with time, some of these channels became inactive. Recordings were made from single patches over periods of up to 45 minutes. Kinetic analysis of these D-glutamate receptor channels is currently underway.

3

REFERENCES

1 Cull-Candy, S G and Usherwood, P N R (1973)
Two Populations of L-glutamate Receptors on Locust Muscle Fibres.
Nature New Biol. 246,62-64.

2 Kerry, C J , Kits, K S, Ramsey, R L, Sansom, M S P and Usherwood, P N R (1987) Single Channel Kinetics of a Glutamate Receptor.
Biophys J 51,137-144.

3 Kerry, C J, Ramsey, R L, Sansom, M S P, Usherwood, P N R and Washio, H (1987) Single Channel Studies of the Action of (+) - Tubocurarine on Locust Muscle Glutamate Receptors. J Exp Biol 127,121-134.

4 Dudel, J, Franke, C, Hatt, H, Ramsey, R L and Usherwood, P N R (1988) Rapid Activation by Glutamate of Excitatory, Cation-Selective Channels in Locust Muscle. Neurosci. Letters (in press).

5 Dudel, J, Franke, C, Hatt, H and Usherwood P N R (1988)
Chloride Channels Gated by Glutamate Receptors (H-Receptors) on Locust Leg Muscle (Submitted to Brain Res).

6 Duce, J A, and Usherwood, P N R (1986)
Primary Cultures of Muscle from Embryonic Locusts (Locusta Migratoria, Schistocerca Gregaria): Developmental, Electrophysiological and Patch-Clamp Studies. J Exp Biol 123,307-323.

4

7 Evans, M L and Usherwood P N R (1985)
The Effect of Lectins on Desensitisation of Locust Muscle Glutamate Receptors. Brain Res 358, 34-39.

8 Lea, T J and Usherwood P N R (1973)
The Site of Action of Ibotenic Acid and the Identification of Two Populations of Glutamate Receptors on Insect Muscle Fibres. Comp Gen Pharmacol. 4, 333-350.

This work was supported by SERC GRANT NO GR/D 67422

[^{3}H]-5-HYDROXYTRYPTAMINE UPTAKE BY NEURONES FROM EMBRYONIC COCKROACH BRAIN GROWING IN PRIMARY CULTURES.

Isabel Bermudez, and D. J. Beadle,

School of Biology and Molecular Sciences, Oxford Polytechnic, Headington, Oxford OX3 0BP, U. K.

5-hydroxytryptamine (5-HT) is widely distributed in the insect central nervous system where it may act as a transmitter[1]. It is well substantiated that vertebrate tryptaminergic neurones possess specific, sodium-dependent transport mechanisms for 5-HT[2]. We have, therefore, investigated the ability of insect central neurones to accumulate 5-HT from the extracellular medium via a sodium-dependent mechanism. We describe here some of the properties of [^{3}H]-5-HT uptake by cockroach central neurones grown *in vitro*. These cultured cells are known to possess receptors for 5-HT[3].

Neuronal cultures were prepared from the cerebral ganglia of 23-day old *Periplaneta americana* embryos and grown in a foetal calf serum-containing medium as described by Beadle *et al*[4]. After 18-20 days of growth *in vitro* cultures were incubated for 30 min in total uptake (TU) saline (200 mM NaCl, 3.1 mM KCl, 9 mM $CaCl_2$, 3.1 mM Tris-HCl; pH 7.2) containing 10 μM pargyline and 1 μM [^{3}H]-5-HT (spec. act. 14.5 Ci/mmol). After incubation the cultures were rinsed in TU saline and then fixed in glutaraldehyde for 30 min at 4°C. The effect of sodium ions and low temperature on [^{3}H]-5HT uptake was investigated by performing uptake in

a sodium-free saline and at 0°C respectively. The effect of 1 μM imipramine and 1 μM 5,6-dihydroxytryptamine (5,6-DHT) was examined by including these substances in the incubation saline.

The fixed cultures were coated in Ilford K_5 emulsion. After 20 days exposure the autoradiographs were developed and then examined with both bright-field and phase contrast optics.

Autoradiographs of the neuronal cultures showed that approximately 30% of the cultured neurones accumulated tritiated 5-HT from the extracellular medium above background levels. [^{3}H]-5-HT labelling appeared to be highly specific as labelled and unlabelled cells were seen in close proximity. [^{3}H]-5-HT uptake was reduced to background levels by sodium-free saline and low temperature. [^{3}H]-5HT accumulation was also reduced by imipramine and 5,6-DHT, two known inhibitors of 5-HT uptake by mammalian tryptaminergic neurones[8].

Our results suggest that a small population of the cultured cockroach neurones are endowed with sodium- and temperature-dependent carriers for 5-HT. The possibility of using these carriers as autoradiographic markers for the identification of subpopulations of cockroach cultured neurones is currently being investigated. The susceptibility of cockroach neuronal uptake of 5-HT to inhibition by imipramine and 5,6-DHT was similar to that reported for mammalian tryptaminergic neurones, suggesting similarities in the structural requirements of the insect and mammalian 5-HT carriers.

The authors thank CIBA-GEIGY Ltd., Basel, for financial support during the course of this work.

1. Nassel, D. R. Prog. in Neurobiol. 1987, 30, 1-85.

2. Kuhar, M. J., Roth, R. H. and Aghajanian, G. K. J. Pharmacol. Exp. Ther. 1972, 181, 36-45.

3. Neumann, R., Lees, G., Beadle, D. J. and Benson, J. A. Sites of Action for Neurotoxic Pesticides (Hollingworth, R. M. and Green, M. B., Eds.), American Chem. Soc., Washington, D. C., 1987, pp. 25-43.

4. Beadle, D.J., Hicks, D. and Middleton, C. J. Neurocytol. 1982, 11, 611-626.

5. Fonnum, F., Lund Karlsen, D., Malthe-Sorenssen, Sterri, S. and Walaas, I. The Cell Surface and Neuronal Function (Cotman, C. W., Poste, G. and Nicolson, G. L., Eds.), Elsevier/North-Holland Biomed. Press, 1980, pp. 455-504.

THE DDT/PYRETHROID RECOGNITION SITE OF THE VOLTAGE-SENSITIVE SODIUM CHANNEL: INTERACTIONS WITH ALKALOID ACTIVATORS AND SEA ANEMONE TOXIN

Jeffrey R. Bloomquist and David M. Soderlund

Department of Entomology, New York State Agricultural Experiment Station, Cornell University, Geneva, NY 14456 USA

Abundant electrophysiological evidence suggests that the voltage-sensitive sodium channel is the primary target site for DDT and pyrethroid insecticides.[1] However, complementary biochemical studies are needed to expand our understanding of the interactions of these insecticides with their binding domain on the sodium channel. Application of radiotracer methods to this problem have demonstrated pyrethroid-dependent enhancement of $^{22}Na^{+}$ flux through chemically-activated sodium channels.[2,3] In order to better understand the nature of these pyrethroid effects in sodium uptake assays and their relationships to mechanisms of acute toxicity, we have undertaken a study of the interactions of DDT and pyrethroid insecticides with alkaloid-dependent activation of sodium channels in mouse brain synaptosomes.[4]

In survey experiments, saturating concentrations of DDT, cismethrin, and deltamethrin enhanced sodium uptake stimulated by veratridine and batrachotoxin, but inhibited uptake stimulated by aconitine. Concentration-response curves for aconitine run in the absence and presence of 10 μM cismethrin demonstrated that the inhibition was noncompetitive. This unanticipated inhibitory effect of insecticides on aconitine-dependent sodium uptake reveals unique characteristics of the interactions of aconitine with the activator recognition site that are not shared by veratridine and batrachotoxin. Studies of the effects of insecticides on veratridine- or batrachotoxin-stimulated uptake found small insecticide-dependent increases in the potency of these activators. In addition to this effect, DDT and deltamethrin, but not cismethrin, enhanced maximal uptake stimulated by veratridine. These findings show that insecticide-dependent modifications of activator potency and

efficacy represent two separate effects that may vary independently with the structure of the insecticide ligand.

Additional experiments were designed to assess the interactions of insecticides and toxin II of the sea anemone *Anemonia sulcata* (ATX II) as modifiers of alkaloid-dependent uptake. DDT and ATX II acted synergistically to increase uptake stimulated by veratridine. Moreover, DDT shifted the potency of ATX II for enhancing veratridine-dependent uptake to five-fold lower concentrations. In contrast, DDT and subsaturating concentrations of ATX II acted independently in their enhancement of sodium channel activation by batrachotoxin. Mutually exclusive effects on veratridine-dependent uptake were observed when cismethrin was coapplied with ATX II. However, independent effects of cismethrin and ATX II were found with aconitine-modified channels, in that cismethrin was able to inhibit ATX II-enhanced aconitine-dependent sodium flux. Thus, the interactions between insecticides and ATX II as modifiers of alkaloid-dependent uptake are complex and depend on the insecticide-activator combination under study.

1. Lund A. E. and Narahashi, T. Pestic. Biochem. Physiol. 1983 20 203-216.
2. Jacques, Y., Romey, G., Cavey, M. T., Kartalovski, B. and Lazdunski, M. Biochim. Biophys. Acta 1980 600 882-897.
3. Ghiasuddin, S.M. and Soderlund, D.M. Pestic. Biochem. Physiol. 1985 24 200-206.
4. Bloomquist, J. R. and Soderlund, D. M. Mol. Pharmacol. (submitted).

THE EFFECTS OF DOPAMINERGIC AGENTS ON AN IDENTIFIED COCKROACH INHIBITORY MOTONEURONE

JULIAN P L DAVIS & ROBERT M PITMAN

Dept. of Biology & Preclinical Medicine, Gatty Marine Laboratory,

University of St Andrews, St Andrews, Fife, Scotland.

Catecholamines have been detected in the nervous systems of several insect species. In the ventral nerve cord of the cockroach (*Periplaneta americana*, *L.*), the dopamine content is approximately 9-10 times that of noradrenaline, while adrenaline is indetectable.[1]

Intracellular current-clamp and voltage-clamp recordings have been made from the soma of a prothoracic common inhibitory motoneurone (designated D_3 by Iles[2]); the preparation was set up in a manner similar to that previously described for the metathoracic ganglion[3].

Intracellular current-clamp recordings show that the application of dopamine to the soma of this motoneurone causes depolarization associated with a modest fall in input resistance[4]. More recent voltage-clamp studies have shown that the response to dopamine is very voltage-dependent. At holding potentials close to the normal resting potential (about -60 mV), dopamine produces a relatively small inward current; at holding potentials progressively more negative than -100 to -150 mV the magnitude of the current increases dramatically. At holding potentials positive relative to the resting potential, the inward current increases more gradually, then decreases, and finally reverses (at approximately 0 mV). The functional significance of this unusual current-voltage relationship is as yet unclear, but might indicate that dopamine is having a predominantly modulatory action. The neurone is also depolarized by the dopaminergic agents ergotamine and apomorphine. Other agents, including octopamine, noradrenaline, and acetylcholine, also cause a depolarization. 5-hydroxytryptamine produced no detectable response.

The action of octopamine has a similar voltage-dependence to that of dopamine, while that of acetylcholine shows very little voltage-dependance. Noradrenaline, on the other hand, has a current-voltage relationship similar to that of dopamine at potentials negative relative to the resting potential, but no inward current is seen at potentials positive relative to the resting potential. The response instead reverses at approximately -50 mV, and the outward current then increases in magnitude at potentials progressively more positive relative to the resting potential.

A range of antagonists have been tested on the preparation to determine whether the response to dopamine involves a specific dopamine receptor, and if so, whether it has characteristics similar to mammalian D_1 or D_2 receptors[5]. The cholinergic antagonists gallamine and α-bungarotoxin have been tested to ascertain whether the response to dopamine could be the result of an interaction with acetylcholine

receptors. Both antagonists suppressed acetylcholine responses, but had no effect on dopamine responses. Metoclopramide blocked octopamine responses, having no effect on dopamine responses. This antagonist has been applied to the locust extensor-tibiae muscle, where it blocks potentiation of twitch tension and the increase in the level of cyclic-AMP, both mediated by octopamine[6,7]. Ergotamine blocked noradrenaline responses, leaving dopamine responses unaffected. The effects of a range of antagonists to mammalian D_1 and D_2 receptors have been studied (see Table 1); dopamine responses could be blocked by both D_1 and D_2 antagonists.

Table 1. Effects of dopaminergic antagonists on dopamine responses in the soma of a cockroach prothoracic common inhibitory motoneurone.

Antagonist	Mammalian target receptor	Action on Insect Inhibitory motoneurone
Fluphenazine (10^{-6}M)	D_1/D_2	+
(+)-Butaclamol (10^{-7}M)	D_1/D_2	+
cis-Flupenthixol (10^{-9}M)	D_1	+
SCH 23390 (10^{-6}M)	D_1	+
Metoclopramide (>10^{-4}M)	D_2	-
Spiroperidol (10^{-5}M)	D_2	+*
Haloperidol (10^{-9}M)	D_2	+
YM 09151-2 (10^{-6}M)	D_2	+

+ = reversible block - = no effect +* = irreversible block

These observations indicate that dopamine responses recorded from the prothoracic common inhibitory motoneurone are unlikely to be due to the interaction of dopamine with receptors for acetylcholine, octopamine, or noradrenaline, but probably involve specific receptors for dopamine. Agents which interfere with cyclic nucleotide metabolism have no effect on dopamine responses or the electrical properties of the neurone[4], and it is therefore unlikely that dopamine produces its effect through a rise in the level of cyclic-AMP. Dopamine responses are blocked by antagonists selective for both mammalian D_1 and D_2 receptors; this insect receptor, therefore, does not appear to conform to the classification of Kebabian and Calne[5].

1 Dymond G R, & Evans P D (1979): Insect Biochem. 9: 535-545.
2 Iles J F (1976): Phil. Trans. R. Soc. Lond., B276: 205-219.
3 Pitman R M (1975): J. Physiol., Lond. 247: 511-520.
4 Pitman R M, & Fleming J R (1985): Pesticide Science 16: 477-448.
5 Kebabian J W, & Calne D B (1979): Nature, Lond. 277: 93-96.
6 Evans P D (1981): J. Physiol., Lond. 318: 99-122.
7 Evans P D (1984): J. Physiol., Lond. 348: 307-324.

OCTOPAMINE RECEPTOR SUBTYPES IN LOCUST SKELETAL MUSCLE: ACTIONS OF OCTOPAMINE AND SYNEPHRINE STEREOISOMERS

Peter D. Evans[1], C. Mohan Thonoor[2] and John M. Midgley[2]

[1]AFRC Unit, Dept. of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ. and [2]Dept. of Pharmacy, University of Strathclyde, Glasgow, Scotland, G1 1XW.

In invertebrate nervous systems specific octopaminergic neurones have been identified, together with target sites for the action of their released octopamine[1]. Many of these target sites possess specific octopaminergic receptors with different pharmacological properties to vertebrate alpha and beta adrenergic receptors[2].

Neuromuscular transmission mediated by the slow excitatory motor neurone (SETi) to the extensor-tibiae muscle of the metathoracic hindleg of the locust is modulated by both presynaptic and postsynaptic octopamine receptors[3] which have been designated $\mathrm{OCTOPAMINE}_{2A}$ and $\mathrm{OCTOPAMINE}_{2B}$ receptors respectively[2]. Drugs that affect the $\mathrm{OCTOPAMINE}_{2A}$ receptors increase the amplitude of SETi-induced twitch tension and drugs that affect the $\mathrm{OCTOPAMINE}_{2B}$ receptors increase the rate of relaxation of SETi-induced twitch tension. In addition the myogenic rhythm of contraction and relaxation found in a proximal bundle of muscle fibres in this muscle is reduced in frequency when $\mathrm{OCTOPAMINE}_{1}$ receptors are activated[2].

Octopamine occurs in three different structural isomeric forms, para-, meta- and ortho-octopamine, all of which have been shown to occur naturally in vertebrates[4]. In insects only p-octopamine has been unequivocally demonstrated to be naturally occurring[5] but m-octopamine has been claimed to be synthesised from L-dopa[6]. Here we describe the activities of the stereoisomers of m-,p- and o-octopamine and of m- and p-synephrine on the different subtypes of octopamine receptor present in the extensor-tibiae muscle of the locust hindleg.

The rank order of potency of the (-)-forms on the $\mathrm{OCTOPAMINE}_{2A}$ receptors was p-synephrine > p-octopamine > m-octopamine > o-octopamine > m-synephrine, whilst the rank order of the (+)-forms was p-synephrine > p-octopamine > m-octopamine. (+)- m-synephrine and (+)- o-octopamine had no effect on this class of receptor when tested up to a concentration of 10^{-3}M.

The rank order of potency of the (-)-forms on the $\mathrm{OCTOPAMINE}_{2B}$ receptors was p-synephrine > p-octopamine > m-synephrine > m-octopamine = o-octopamine whilst the rank order of the (+) forms was p-octopamine > p-synephrine > m-octopamine > o-octopamine. (+)- m-synephrine again had no

effect up to a concentration of 10^{-3}M.

The rank order of potency of the (-)-forms on the $OCTOPAMINE_1$ receptors was p-synephrine > p-octopamine > m-synephrine > m-octopamine > o-octopamine, whilst the rank of the (+)-forms was p-synephrine > p-octopamine > o-octopamine > m-synephrine > m-octopamine.

In all cases the (-) stereoisomeric forms of all the structural isomers tested were more potent than the (+)- forms with isomeric activity ratios varying from 2-2325. In general all the isomeric forms of octopamine and synephrine tested were much more potent agonists of $OCTOPAMINE_1$ receptors than $OCTOPAMINE_2$ receptors. This was particularly noticeable for the m-isomers, where (-)- m-octopamine, for example, was 30,000 and 5,000 times more potent on $OCTOPAMINE_1$ than on $OCTOPAMINE_{2A}$ and $OCTOPAMINE_{2B}$ receptors respectively. The isomeric forms of octopamine and synephrine differ in the rank order of potency on insect octopamine receptors from that found for alpha and beta adrenergic receptor subtypes in vertebrates[7,8]. On vertebrate adrenergic receptor subtypes meta isomers are in general more potent than the corresponding para-isomers whereas the converse is always true of insect octopamine receptors. This suggests that the rank order of potency of the different stereoisomeric forms of the structural isomers of octopamine and synephrine may be of use in the differentiation of specific octopamine receptors from adrenergic receptors in the vertebrate nervous system.

1. Evans, P.D. Adv. Insect Physiol. 1980, 15, 317-473.
2. Evans, P.D. J. Physiol. 1981, 318, 99-122.
3. Evans, P.D. and O'Shea, M. Nature (Lond.) 19 270, 257-259.
4. Williams, C.M., Couch, M.W., Thonoor, C.M. and Midgely, J.M. J. Pharm. Pharmacol. 1987, 39, 153-157.
5. Goosey, M.W. and Candy, D.J. Insect Biochem. 1980 10, 393-397
6. David, J.C., Coulon, J.F., Lafon-Cazal, M. and Vinson, D. Experientia 1981, 37, 804-805.
7. Brown, C.M., et al., Brit. J. Pharmacol. 1988 (in press)
8. Jordan, R., Midgley, J.M., Thonoor, C.M. and Williams, C.M. J. Pharm. Pharmacol. 1987, 39, 752-754.

DELTAMETHRIN RAISES POTASSIUM ION ACTIVITY IN THE MICRO-ENVIRONMENT OF THE CENTRAL NERVOUS SYSTEM OF AN INSECT, *Periplaneta americana*

Christopher H. Hendy and Mustafa B.A. Djamgoz

Department of Pure and Applied Biology, Imperial College, London SW7 2BB.

Pyrethroid poisoning of insects has been shown frequently to involve repetitive spike activity in the intact central nervous system (CNS).[1] In isolated axons of the cockroach, deltamethrin (DM) was found to cause sodium-dependent, tetrodotoxin-sensitive depolarization of membrane potentials[2], whilst *in vivo* it induced repetitive firing leading to conduction block.[3] However, ionic events occurring within the CNS *micro-environment* during pyrethroid action are not known. This study reports effects of DM on extra-neuronal K^+ activity, $[K^+]_e$, in the ventral nerve cord (VNC) of the cockroach.

Experiments were performed on a dorsally-dissected cockroach preperation described earlier.[3,4] $[K^+]_e$'s were measured using double-barrelled K^+-sensitive micro-electrodes based on a neutral ion-carrier[4]; spontaneous activity in the VNC was monitored using a suction electrode. DM was used at 10 um as a fast-acting working concentration.[3]

At normal room temperature (around 20°C), DM-induced increase in neural activity was followed by a gradual rise in $[K^+]_e$. The time course of the latter effect was more or less linear over 35 minutes; during this period $[K^+]_e$ increased from the resting value of 4.5 mM to about 7.5 mM. We have previously shown that at this value of $[K^+]_e$, neural activity would not normally be affected.[4] Upon cooling the preparation to 8°C, $[K^+]_e$ rose to around 6.4 mM. When DM was subsequently applied, $[K^+]_e$ gradually increased by a further 1.2 mM to 7.6 mM over 20 minutes and remained steady at this

value. Spontaneous activity was mostly suppressed. There was no significant difference between the final values of $[K^+]_e$ reached 35 minutes after DM treatment at 20^oC vs. 8^oC. Thus, suppressing metabolic ion pumping by cooling does not seem to affect the DM-induced rise in $[K^+]_e$. When the structural component of the blood-brain barrier was destructed by application of 3 M urea for 15 seconds[5], $[K^+]_e$ initially rose, then fell to around 4 mM. After DM treatment, $[K^+]_e$ remained unchanged for about 10 minutes, then rose over the following 25 minutes to 4.4 mM. Spontaneous activity increased transiently before conduction block was achieved. However, the latter increase was considerably less than normal, although conduction block occurred over approximately the same time course. It was clear that spontaneous activity bursts preceeded the rise in $[K^+]_e$.

We conclude that the insect blood-brain barrier is involved to a limited extent in shaping the action of DM on cockroach axons. However, the metabolic component of the barrier does not appear to be the primary cause of the well-known negative temperature coefficiency of pyrethroid action.[6]

REFERENCES

1 Casida, J.E., Gammon, D.W., Glickman, A.H. and Lawrence, L.J. A.Rev.Pharmacol.Toxicol. 1983, 23 413-438.

2 Laufer, J., Roche, M., Pelhate, M., Elliott, M., Janes, N.F. and Sattelle, D.B. J.Insect Physiol. 1984, 30 341-349.

3 Hendy, C.H. and Djamgoz, M.B.A. J.Pestic.Sci. 1985, 16 520-529.

4 Hendy, C.H. and Djamgoz, M.B.A. J.exp.Biol. 1987, 127 211-227.

5 Treherne, J.E., Schofield, P.K. and Lane N.J. J.exp.Biol. 1973, 59 711-723.

6 Wang, C.M., Narahashi, T. and Scuka, M. J.Pharmacol.exp.Ther. 1972, 182 442-453.

STUDIES INVOLVING AVERMECTIN AND THE GABA RECEPTOR OF ASCARIS MUSCLE.

Lindy Holden-Dye, Graham M. Hewitt, Kenneth T. Wann[*], Povl Krogsgaard-Larsen[+] & Robert J. Walker.

Dept. of Neurophysiology, School of Biochemical & Physiological Sciences, Bassett Crescent East, Southampton University, Southampton S09 3TU; [*]Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ and [+]Royal Danish School of Pharmacy, Universitets Parken 2, DK-2100 Copenhagen, Denmark.

Despite considerable evidence for GABA as the inhibitory transmitter onto Ascaris body wall muscle cells [1-3] there have been only limited studies on the pharmacology of the GABA receptor involved. Piperazine has been shown to mimic the action of GABA on these cells and probably acts on the same receptor [1,4]. The anthelmintic Avermectins, first isolated ten years ago[5], have been shown to alter the characteristics of the GABA single channel current pulses[7] in *Ascaris* muscle cells. The aim of the present study is to characterise the *Ascaris* muscle GABA receptor and investigate the action of avermectin on these cells.

Intracellular recordings were made from both silent and spontaneously active cells in *Ascaris* muscle body wall maintained at 32 or 37^{o}C in artificial perienteric fluid (APF). In the case of the silent cells, a second intracellular electrode was inserted to inject current pulses and enable determination of membrane conductance. Bioelectric potentials were amplified using conventional electrophysiological recording techniques and displayed on a Hewlett-Packard pen recorder. Muscle cell membrane potentials were usually in the range -30 to -40mV. The action potentials recorded from active cells were up to 40 mV amplitude. Drugs were applied by perfusion over the preparation at a rate of 7 ml min-1 for up to 3 min. In some experiments GABA was applied locally to the cells by pressure ejection using a Picospritzer (1 mM in pipette).

Hill analysis of concentration response curves for cell hyperpolarization to bath applied GABA gave an EC 50 of 12.6 +/- 0.2 µM and a Hill coefficient of 2.2 +/- 0.2 (n=5, +/- SEM). The Hill coefficient is consistent with the hypothesis that up to 2 GABA molecules are required to activate the GABA receptor. GABA 10 µM increased input conductance by up to 2.3 µS against a mean resting input conductance of 2.57 +/- 0.17 µS (n=17). The mean GABA induced increase in input conductance as a percentage of control was 34 +/- 8%. The GABA response is dependent on external chloride concentration and is independent of changes in external sodium and potassium.

A range of GABA receptor agonists was applied by perfusion. The relative potency compared to GABA was calculated as the ratio of a GABA concentration to an equipotent concentration of agonist, from parallel portions of the dose response curve [TABLE 1]. Bath applied bicuculline methiodide, picrotoxin, securinine, pitrazepin and SR 95331 were unable to antagonise the hyperpolarization resulting from pressure ejection of 1 mM GABA onto the muscle cells.

Avermectin (MK 936 : 80:20 mixture of Avermectin B-1a and Avermectin B-1b) was tested on silent cells, against the GABA response and on the membrane potential or conductance of quiescent cells. Avermectin (1 μM) irreversibly reduced the response to pressure ejected GABA by about 25% (n=17). Avermectin (1 μM) blocked action potentials in spontaneously active muscle cells. This block was unlike the fast inhibition of spontaneous activity seen with GABA application since Avermectin block developed after 26 +/- 6 min. (n=7) and was not accompanied by membrane hyperpolarization. Avermectin failed to exert a block of spontaneous activity when the cells were perfused with APF in which acetate ions have been substituted for chloride ions. However it still exerted a block in the presence of 10 μM picrotoxin.

This pharmacological profile for the GABA response of *Ascaris* muscle cells indicates that the receptor involved does not readily fit into the vertebrate classification. This study demonstrates that Avermectin blocks, with a slow time course, the GABA response in quiescent cells and action potentials in spontaneously active cells in a chloride dependent, picrotoxin insensitive manner. Further studies are in progress to determine the extent to which these properties of Avermectin contribute towards its anthelmintic action.

TABLE 1.

RELATIVE POTENCIES OF COMPOUNDS ON *ASCARIS* MUSCLE BAG CELL COMPARED TO GABA INDUCED HYPERPOLARIZATION.

Drug	Relative Potency	(n=)
GABA	1	
trans-4 AMINOCROTONIC ACID	0.55 ± 0.03	(6)
MUSCIMOL	0.25 ± 0.13	(5)
IMIDAZOLE 4-ACETIC ACID	0.20 ± 0.03	(4)
ISOGUVACINE	0.19 ± 0.07	(3)
GUANIDO ACETIC ACID	0.16 ± 0.02	(4)
β-GUANIDOPROPRIONIC ACID	0.085 ± 0.017	(3)
δ-AMINOVALERIC ACID	0.079 ± 0.040	(3)
cis-4 AMINOCROTONIC ACID	0.031 ± 0.017	(3)
THIP	0.006 ± 0.001	(3)

3-aminopropanesulphonic acid, piperidine-4 sulphonic acid, baclofen, glycine, β-alanine, taurine were without effect up to 1 mM.

Acknowledgement. We are grateful to the SERC for financial support and to Sanofi Recherche for a supply of SR 95331.

1. del Castillo, J., de Mello, W.C., Morales, T.A. *Experimentia* 1964a, 20, 141-145.
2. Martin, R.J. Brit. *J. Pharmacol*. 1980, 68, 128-129.
3. Johnson, C.D. & Stretton, A.O.W., *J. Neuroscience* 1987, 7, 223-235.
4. Martin, R.J. *Brit. J. Pharmacol*. 1982, 77, 255-265.
5. Burg, R.W., Miller, B.M., Baker, E.E., Birnbaum, J., Currie, J.A., Hartman, R., Kong, Y-L., Monaghan, R.L., Olson, G., Putter, I., Tunac, J.P., Wallick, H., Stapely, E.O., Oiwa, R., Omura, S. *Antimicrob. Agents Chemother*. 1979, 15, 361-367.
6. Martin, R.J. *Biochem. Soc. Trans*. 1987, 15, 61-65.
7. Brading, A.F., Caldwell, P.C. *J. Physiol. Lond*., 1971, 217, 605-624.

THE INFLUENCE OF OXYGEN SUPPLY ON INSECT MUSCLE RESTING POTENTIAL

Leszek Janiszewski and Barbara Grajpel
Department of Animal Physiology, Institute of Biology,
N.Copernicus University, Toruń, Poland.

Ikeda and Kaplan were able to show that the proper oxygen supply of the tracheal system influences the activity pattern of the neurons in Drosophila melanogaster[1].Ikeda /personal comunication,1985/was succesful to maintain the resting potential of the body wall muscle of larval Drosophila at 80 mV level for many hours. Without proper air supply this resting potential went down to 30mV.The purpose of the present study was to check the effect of oxygen supply on the resting potential of the muscles of larval Tenebrio molitor.

Abdominal longitudinal ventral muscles were used for the measurements of muscle resting potential.Details concerning rearing of animals and making the preparation are given in previous paper[2].The insect prepared for the experiment was placed in a special chamber filled with physiological solution,which had the following composition/in mmoles/:80NaCl, 40KCl,5$CaCl_2$, 10$MgCl_2$and 435 glucose[3].The construction of the chamber allowed the air to supply the tracheal system of the insect during the experiments. The experiments were performed in two series :first without air and the second with air supply. In each series 8 animals were used.The measurements were performed at 0,20,30,40,50 and 80 minutes.In each above mentioned experimental point 10 measurements were performed on each animal.Each point on the graph/Fig.1/represents the mean value of 80 measurements $\pm$ SE.

The importance of oxygenation of the physiological saline in microelectrode experiments arises in many experimental arrangements[4].It may be seen from the present results of the experiments with air supply of the preparation that this supply through the tracheal system exerts a marked influence on the muscle resting potential.These results point out the importance of the concept of the complex nature of the genesis of the muscle resting potential[5].This problem concerns not only

the ions taking part in the origin of resting potential,but also the question of looking at the resting potential as a sum of the ionic diffusion potential predicted by Nernst equation or constant field equation and of the potential produced by the electrogenic pump.

The obtained results indicate the necessity of further experiments in two directions:quantitative estimation of the amount of oxygen in the air which supplies the preparation /ev. changing the amount of oxygen/and investigating the possible mechanisms of the oxygen action.

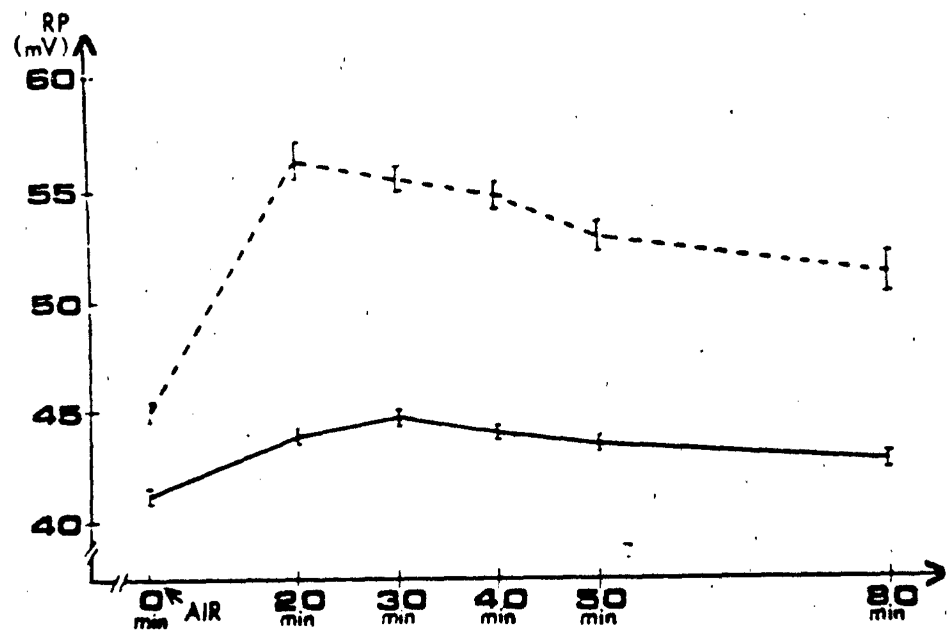


Fig.1 The changes of the muscle resting potential in the larva of Tenebrio molitor.Ventrical lines represent the standard errors.

1.Ikeda,K.,Kaplan,W.D. Amer.Zool.1974,14, 1055-1066

2.Janiszewski,L.,Grajpel,B.Gen.Physiol.Biophys.1985,4,537-540

3.Kusano,K.,Janiszewski,L.Electrobiology of Nerve,Synapse and Muscle.Raven Press,New York,1976,95-103

4.Vyskočil.F.,Di Gregorio,F.,Gorgio,A. Pflügers Arch.1985, 403,1-6

5.Akaike,N. J.Physiol.1975,245,499-503

PYRETHROID MODIFIED SODIUM CHANNELS IN SNAIL NEURONES

Tibor Kiss

Balaton Limnological Research Institute of the
Hungarian Academy of Sciences, H-8237 Tihany, Hungary

A theory of pyrethroid action was proposed according to which both normal and drug modified channels exist simultaneously in the membrane.[1] Modified channels activate and inactivate slower than normal ones. It was, therefore suggested that pyrethroids interact with the various kinetic states of Na-channel in all cases slowing channel kinetics.[2] Upon application of pyrethroids, however, never was observed modification of all available voltage-dependent Na-channels, only fraction of them. It can be supposed, therefore that pyrethroids binding to the Na channel promote to open the channel into a second conducting state. Presence of the second open state of the Na-channel in normal conditions was reported on neuroblastoma[3] and heart cells.[4] Here we bring some evidences supporting this idea.

Effect of deltamethrin was studied on the Na-channels of identified snail /Helix pomatia L./ neurones under voltage-clamp conditions. At 5×10^{-5}M concentration deltamethrin decreased the resting membrane potentials, amplitude of action potentials and increased the negative afterpotentials. The main effect appeared as the marked slowing down of the inactivation of the Na-currents. The inactivation curve was shifted to the depolarizing direction by 10-15 mV, which is different from the results obtained by other pyrethroids.[1,5]

In control saline the inactivation of Na-current could be fitted by one exponential, but sometimes by two. However,

after deltamethrin treatment Na-current relaxed always along two exponentials. The fast / 5-10 ms/ and slow / 35-40 ms/ time constants were comparable to time constants measured in control saline.
The semilogarithmic plot of tail currents in the presence of deltamethrin revealed two time constants, which were similar in order of time constants of inactivation of Na-current. The recovery kinetics from inactivation in control and deltamethrin treated neurones were similar.
On the basis of our results a kinetical model is suggested according to the pyrethroid increases the possibility of Na-channel to open into a second conducting state, then inactivate into the same state but with different rate constants.

1. Lund, A.E., Narahashi, T. Neurotoxicology. 1981 2 213-229
2. Chinn, K., Narahashi, T. J.Physiol. 1986 380 191-207
3. Nagy, K., Kiss, T. and Hof, D. Pflügers Arch. 1983 399 302-308
4. Kunze, D., Lacerda, A.E., Wilson, D.L. and Brown A.M. J.gen.Physiol. 1986 86 691-719
5. Lund, A.E., Narahashi, T. J.Pharmac.Exp.Therap. 1981 219 464-473

EFFECT OF DIHYDROAVERMECTIN B_{1a} ON Cl SINGLE-CHANNEL CURRENTS IN *ASCARIS* MUSCLE

Martin, R. J. and Pennington, A. J.

Department of Preclinical Veterinary Sciences, R(D)SVS, Summerhall, Edinburgh

We have studied effects of Dihydroavermectin B_{1a} (DHAVM 2×10^{-12} to 2×10^{-7}M) on GABA-activated channels[1] and spontaneously active channels in *Ascaris* muscle membranes using outside-out patches and solutions favourable for recording Cl^- currents.

We found no evidence to suggest that DHAVM (2×10^{-7}M) can act as an ionophore; no channel currents were produced after application of DHAVM to the outside of patches which did not contain GABA-activated or spontanously active channels; the patches remained quiet.

We found that DHAVM (2×10^{-12}M) acts on a spontaneously active (circa 10pS) channel to increase the probability of being open (P_{open}): patches which did not contain GABA-activated channels but which contained the channel showed an increase in P_{open} after DHAVM application, Fig. 1.

DHAVM (2×10^{-7}M) affected the properties of GABA-activated channels: after a delay it reduced P_{open} of GABA activated channels and increased the variance of their channel currents; in some patches DHAVM produced an initial increase in P_{open} before the onset of antagonism.

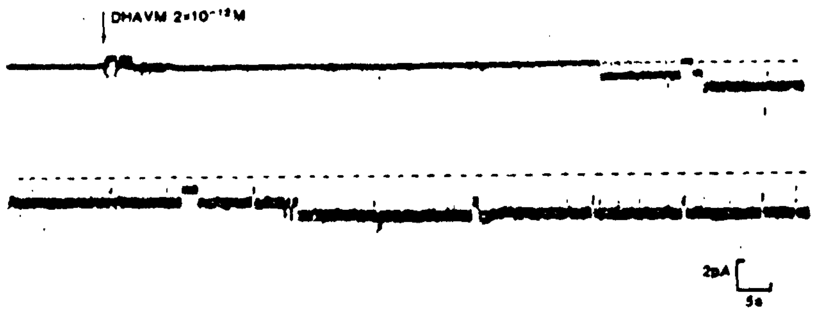
Acknowledgements

A. P. is supported by the SERC. DHAVM was a gift of Pfizer Central Research (Dr K. Gration).

1. Martin, R. J. (1985) Gamma-Aminobutryic acid- and piperazine-activated single-channel currents from *Ascaris suum* body muscle. Br. J. Pharmac. 84, 445-461.

Figure 1 Effect of DHAVM on an outside out patch

2 x 10^{-12}M DHAVM (dissolved in 0.1%DMSO) was added to the bath and thus to the outside of an outside out patch. It dramatically increased P_{open} of spontaneously active channels which have an amplitude of 0.9pA (10pS). Patch potential: -75mV. Pipette solution (mM): 140CsCl; 2$MgCl_2$; 5TRIS; 11EGTA; 1$CaCl_2$; adjusted to pH 7.2. Bath solution (mM): 105 Na acetate; 35 NaCl; 2KCl; 2$MgCl_2$; 5TRIS; 3 Glucose; 5$CaCl_2$. The dashed line represents the closed state. The lower trace continues the upper trace. Up to three channels are open at the end of the lower trace. The effect of DHAVM was not reversed on washing (not shown). Note the slow time scale and the increase in the noise limits as the channels open.



Ion channels formed by a short chain α-helical peptide - Mastoparan.

Ian R. Mellor and M.S.P. Sansom.

Department of Zoology, University of Nottingham, University Park, Nottingham. NG7 2RD.

There has recently been considerable interest in ion channel formation by α-helical peptides such as alamethecin and its derivatives[1]. These are thought to act via formation of α-helix clusters in the membrane[2] and hence are of interest as models of the ionophoric regions of channel proteins from excitable cell membranes. We have investigated two such peptides - *Staphylococcus aureus* δ-toxin[3,4] and mastoparan, both of which are active ingredients of natural toxins, and are composed entirely of naturally occurring L-aminoacids. Mastoparan, from the venom of *Vespula lewisii*, consists of only fourteen amino acid residues:

Ileu-Asn-Leu-lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ileu-Leu-NH_2

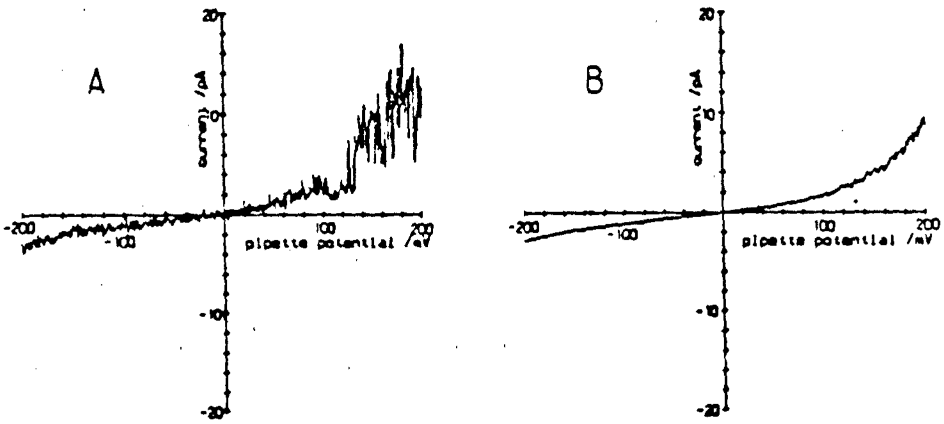
It is therfore expected to form an α-helix of length 2.1nm ie. shorter than the thickness of a lipid bilayer.

The 'pipette dipping method'[5] has been used to record from peptide channels reconstituted in planar lipid bilayers. In this approach, an artificial lipid bilayer is assembled on the tip of a patch pipette. This is done by withdrawing the pipette tip from a lipid monolayer spread at an air/water interface, starting with the tip on the water side, and then lowering it back through the same monolayer. The lipid monolayer is spread by evaporation of pentane from a solution of 1,2-diphytanoyl-phosphatidylcholine in pentane added to the air/water interface. The air/water interface is simply the surface of a bath containing a buffered electrolyte solution.

Mastoparan was added to the solution contained in the patch pipette ie. the *cis* compartment. Initial experiments clearly showed the formation of ion channels at mastoparan concentrations of ca. 0.7μM. Channel activity was more pronounced when the pipette potential was positive. At *cis* +100mV, the channel conductance was ca. 100-150pS, whereas at -100mV, the conductance was only ca. 30pS. Application of voltage ramps from -200 to +200mV across the bilayer, was used to construct current-voltage relationships for mastoparan containing membranes. These were markedly assymmetric, with the channel predominantly closed at negative potentials, and open at positive potentials. The direction of sweep of the ramp ie.

positive to negative or negative to positive, did not appear to alter the shape of the relationship.

FigA. *A current vs. voltage plot for a single voltage ramp sweep.* FigB: *An average I-V plot of 26 such ramps.*



The channel properties of mastoparan will be interpreted in terms of structural models.

References

1. Menestrina,G., Voges,K., Jung,G. and Boheim,G. J. Membrane Biol. 1986, 93 111-132.

2. Freer,J.H. and Birkbeck,T.H. J. Theor. Biol. 1982, 94 535-540.

3. Sansom,M.S.P. and Mellor,I.R. Neurosci. 1987, 22 S685.

4. Mellor,I.R., Thomas,D.H. and Sansom,M.S.P. Biochim. Biophys. Acta. 1988, (submitted for publication).

5. Coronado,R. and Latorre,R. Biophys. J. 1983, 43 231-236.

POTASSIUM CHANNELS IN CULTURED LOCUST MUSCLE.

Barbara A. Miller, and Peter N.R. Usherwood.
Zoology Department, Nottingham University, Nottingham NG7 2RD, U.K.

Primary cultures containing large contractile myofibres were prepared from mechanically dissociated locust embryos (*Schistocerca gregaria*) which had been eviscerated. The cells were plated out in "5+4" medium using a modified version of the hanging column technique [1, 2]. Skeletal myofibres were used for electrophysiological investigations after two months *in vitro*.

Cultures were bathed in standard locust saline (180mM NaCl, 10mM KCl, 2mM $CaCl_2$, 3mM Hepes buffer, pH 6.8). In this saline the myofibres had a mean resting potential of -39 ± 7mV (n=42). Pipettes, made of haemocrit tubing, were shank-coated with Sylgard resin but were not polished. They contained either standard locust saline or a K^+-rich saline in which the concentrations of Na^+ and K^+ in the standard saline were reversed. Experiments were conducted at 20-22°C. A List patch-clamp amplifier (LM-EPC7) was used in the voltage-clamp mode to record single channel currents. The currents were recorded on a video-cassette recorder (Sony β-max S1 F30) using a pulse-code modulation unit (PCM 701 ES) modified to give a uniform frequency response from DC to 20KHz [3].

About 60% of the seals between the tip of a patch pipette and a cultured myofibre formed spontaneously when the pipette tip touched the surface of the myofibre. The remaining seals were obtained by applying a slight amount of suction to the pipette interior. Cell-attached, inside-out and outside-out patches were used in this study [4]. Channels were often seen as soon as a seal was formed. Unitary current events, representing the activity of a single channel in a patch, being observed in almost half of the patches obtained.

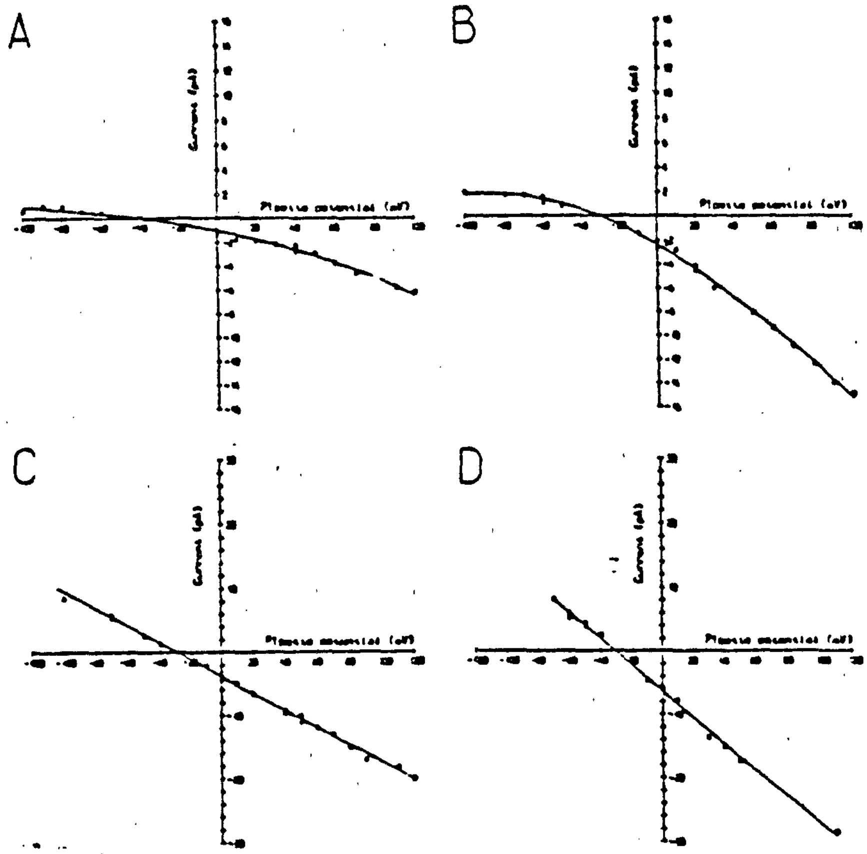
Five types of channel have been identified in cell-attached and excised patches; these have been designated Type-1 to -5. At least four of these are thought to be K^+ channels [5]. The activities, conductances and reversal potentials of these channels were unaffected by the Cl^- channel blocker picrotoxin or by substitution of SO_4^{2-} for Cl^- in either the pipette and/or the bathing saline.

When recordings were obtained from cell-attached patches using high K^+ pipettes the potassium equilibrium potential (E_K) was equal to a pipette potential (Vpip) of -40mV. With high K^+ pipettes the Type-1 channel (Fig.1 A) had a conductance of 43pS for inward currents between Vpip of +100mV and -40mV, and 8pS for outward currents between Vpip -40 and -60mV. This channel exhibited bursting behaviour, with well resolved open and closed states. It had a subconductance state which was 3/4 the full conductance for the channel. Kinetic analysis of this channel revealed at least 4 closed states and at least 3 open states. The Type-2 channel (Fig.1 B) had a chord conductance of 115pS for inward currents between Vpip +100 and -30mV, and 29pS for outward currents between -30 and -160mV. It exhibited a "flickering" pattern of bursting with very brief openings and closings. Subconductance states were observed for the inward current these were 1/4, 1/2 and 3/4 of the full channel conductance. Type-1 and-2 channels do not appear to be Ca^{2+}-sensitive, since exposing excised patches to 2mM Ca^{2+} on the internal and/or the external membrane surface did not seem to effect the activities of these channels. But they were blocked when Ba^{2+} (20mM) was present in the bath saline. The current/voltage (I/V) relationships of the Type-1 and Type-2 channels (Fig.1 A & B) suggest that they are inward rectifiers. However, there is evidence that the probability of these channel being open is greater when the membrane is depolarized so that the overall current flow through the channel may be ohmic. The Type-1 and Type-2 channels accounted for about 90% of the channels seen in patches. Between them they contribute a significant amount of current at the resting potential of the myofibre. The Type-3 channel (Fig.1 C) had a conductance of 130pS. This channel exhibited brief openings in cell-attached patches and its gating kinetics were sensitive to Ca^{2+}. It has at least 3 closed states and at least 2 open states. The Type-4 channel (Fig.1 D) had a conductance of 207pS. This channel was rarely observed, and it did not appear to exhibit any dependence upon Ca^{2+}. It has at least 3 closed states and at least 1 open state. Type-3 and Type-4 channels did not exhibit any rectification (although Goldman rectification was observed in excised patches with asymmetrical salines). The Type-5 channel currents were of very small amplitude being only 2pA at a Vpip of +160mV. This channel had openings and closings of relatively long duration. Because of the poor signal-to-noise ratio the reversal potential for the channel current could not be ascertained, but extrapolation of the I/V curve suggests that this may also be a K^+ channel.

In the adult locust the K^+ channels exhibit a slight Na^+ permeability [6]. We have yet to establish whether this is also true for the embryonic K^+ channels.

Figure 1. Current/voltage relationships for the four types of K^+ channel identified. All the data was obtained from cell-attached patches using pipettes containing K^+-rich saline.
A. Type-1 channel.
B. Type-2 channel.
C. Type-3 channel.
D. Type-4 channel.

FIGURE 1.



1. Chen, J.S. & Levi-Montalcini, R. *Science* 1969, Vol. 166. pp 631-632.

2. Duce, J.A. & Usherwood, P.N.R. *J. Exp. Biol.* 1986, Vol. 123. pp 307-323.

3. Lamb, T.D. *J. Physiol. (London).* 1984, Vol. 102. 5P.

4. Hamill, O.P.; Neher, E.; Sakmann, B. & Sigworth, F.J. *Pflügers Archiv.* 1981, Vol. 391. pp 85-100.

5. Duce, J.A.; Miller, B.A. & Usherwood, P.N.R. in *"Cell Culture Approaches to Invertebrate Neuroscience", D.J. Beadle, G. Lees. & S.B. Kater, Eds., Academic press, London.* 1988. Chapt. 8. pp 221-233. In press.

6. Hoddie, P.L.; Ramsey, R.L. & Usherwood, P.N.R. *J. Physiol. (London).* 1986, Vol. 378. 60P.

SEROTONIN (5-HT_3) RECEPTOR-OPERATED IONIC CHANNELS IN MOUSE NEUROBLASTOMA CELLS.

Hans C. Neijt and Henk P.M. Vijverberg.

Department of Veterinary Pharmacology, Pharmacy and Toxicology, University of Utrecht, P.O. Box 80.176, 3508 TD Utrecht.

In cultured mouse neuroblastoma (N1E-115) cells 5-HT_3 receptors have been demonstrated by electrophysiological as well as radioligand binding studies[1-3]. Using the whole-cell voltage clamp suction pipette technique the properties of the membrane current mediated by 5-HT_3 receptors were investigated by superfusion of entire cells with external solution containing various concentrations of the agonist for adjustable periods.

At a membrane potential of -70 mV 5-HT causes a rapid increase of inward current followed by a decrease. The I/V-curve of this ionic current is linear between -80 mV and 60 mV, with a reversal potential of 20 mV. This indicates that sodium and potassium ions permeate through the ionic channel in a ratio of approximately 2.5:1. The peak amplitude of the inward current depends on the logarithm of the agonist concentration in a sigmoidal way and the half maximum response is induced by 2 μM 5-HT. The kinetics of the ionic current are independent of membrane potential, but are strongly affected by changes of the 5-HT concentration (Fig. 1A).

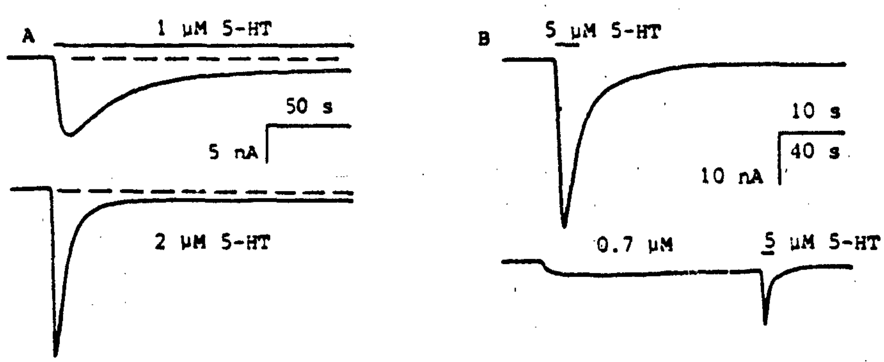


Fig. 1. A. Inward currents evoked in a neuroblastoma cell by whole-cell superfusion with 5-HT at two concentrations indicated. Peak amplitude increases and the kinetics become more rapid at higher concentration. B. Desensitization after pre-exposure to 5-HT. Following exposure to a low concentration of 5-HT the amplitude of the inward current is reduced (lower trace) with respect to control (upper trace).

The time constant of exponential decay of the inward current decreases with increasing 5-HT concentration to a minimum value of 6.5 s for concentrations ≥ 3 μM 5-HT.

Upon repeated application and in the continuous presence of agonists the amplitude of the 5-HT response rapidly decreases as a result of desensitization (Fig. 1B). The concentration-effect curve of steady-state desensitization induced with 5-HT shows an IC_{50} of 0.1 μM 5-HT. The onset of desensitization, induced by pre-exposure to 5-HT for variable periods, follows the same time course as the inward current decay. When the agonist is removed the ionic current rapidly declines, i.e., activation is reversed, and recovery from desensitization also occurs. The time constants of these exponential processes are 6.9 s for the reversal of activation and 18 s for the recovery from desensitization. Both processes are independent of membrane potential and 5-HT concentration.

The results suggest that the decay of the ionic current and the onset of desensitization reflect the same molecular transitions of the $5\text{-}HT_3$ receptor-ionic channel complex. The kinetics of these transitions are limited by the rate of association of the agonist to the receptor at low concentrations and by the conformational changes of the receptor-ionic channel complex at high concentrations. This implies that at the time of peak inward current the agonist receptor interaction has not yet reached equilibrium and that the affinity of the agonist for the $5\text{-}HT_3$ receptor is best described by the steady-state desensitization curve. A cyclic model containing five states of the $5\text{-}HT_3$ receptor-ionic channel complex is proposed to account for the observed kinetic properties of the ionic current.

1. Neijt H.C., Vijverberg H.P.M. and van den Bercken J. European Journal of Pharmacology 1986 127 271-274

2. Neijt H.C., te Duits I.J. and Vijverberg H.P.M. Neuropharmacology 1988, in press

3. Hoyer D. and Neijt H.C. Molecular Pharmacology 1988, in press

IONIC CURRENTS IN AN IDENTIFIED INSECT MOTONEURONE

WENDY D. NIGHTINGALE AND ROBERT M. PITMAN

St Andrews University. Department of Biology and Preclinical Medicine. Gatty Marine Laboratory. St Andrews. KY16 8LB. SCOTLAND

Outward currents in an identified cockroach (*Periplaneta americana*) metathoracic basalar/leg depressor motoneurone (cell 3 [1]) have been studied, using two-electrode voltage-clamp.

Starting from a holding potential of -70mV, a BBC model B microcomputer and interface were used to generate a series of positive steps (110ms duration) which successively incremented by 10mV. The magnitude of the net outward current progressively increased as command steps approached 100mV. As the size of command step was further increased, the evoked currents underwent a decline before continuing to rise (Fig. 1A). Current measurements taken at 50ms produce a N-shaped I-V curve (Fig. 1B) similar to that previously reported in molluscan neurones [2], and another identified insect motoneurone [3]. Individual current traces, produced by command steps between -40 and 50mV, showed a small (< 1uA), fast (< 1ms duration) net inward current that was followed by a strong net outward current which eventually masked the inward component at potentials greater than 50mV (Fig 1C).

Positive command steps from holding potentials more negative than -70mV provided no evidence for an early transient outward current similar to I_A reported in molluscan neurones [4]. Furthermore, the aminopyridines 3,4-diaminopyridine (5mM) and 4-aminopyridine (5mM) had little effect upon the I-V relationship. Therefore, if present at all, I_A contributes very little to the net outward current.

The net outward current could be pharmacologically separated into two major components. The component responsible for the N-shape was readily supressed by drugs and procedures which blocked the action of calcium. The N-relationship was reversibly abolished by cadmium (1mM) or manganese (5mM) ions, and was reversibly reduced by the dihydropyridine blocker verapamil (50uM). Under certain conditions the bee-venom toxin, apamin, also reversibly abolished the N-shape. At concentrations up to 20uM the toxin had no effect upon the N-relationship. If, however, the currents were first supressed by cadmium (1mM), then restored by washout before application of apamin (4uM), the toxin was now able to abolish the N-relationship. The I-V curve was readily restored to normal on washing out the toxin. This suggests that the presence of residual cadmium as a calcium antagonist facilitated the action of apamin in this preparation by decreasing the availability of free calcium-binding sites. The above results indicate a strong calcium-dependent outward current.

The classical I_K blocker tetraethylammonium (TEA^+), when externally applied, reversibly reduced the net outward current and abolished the N-relationship. Pre-treatment with cadmium (1mM) to remove any calcium contribution followed by application of TEA^+ (25mM) produced a further reduction in the net outward current, indicating that TEA^+ acts on both calcium-dependent and calcium-independent components of the outward current.

Lowering the external chloride ion concentration to 47mM (20% of its normal concentration) caused a reduction in the net outward currents. This may be an indirect effect on a conductance to another ion, or a direct effect on chloride ion outward currents.

Tail currents were measured using a pre-pulse followed by a test pulse regime in order to determine the reversal potential for the outward current. Consistent reversal potential measurements were difficult to obtain because these currents were very dependent upon pulse duration (Fig. 1D); increasing the duration of the pre-pulse from 10 to 50 ms resulted in a positive shift in reversal potential. This shift is thought to be due to ion accumulation within the highly infolded neuronal membrane during the pre-pulse [4]. For small pre-pulse durations of 10 to 20ms, and pulse magnitude of 0, 50 and 100mV, the reversal potential lies around -70mV. The most likely ionic species responsible for the outward current is potassium.

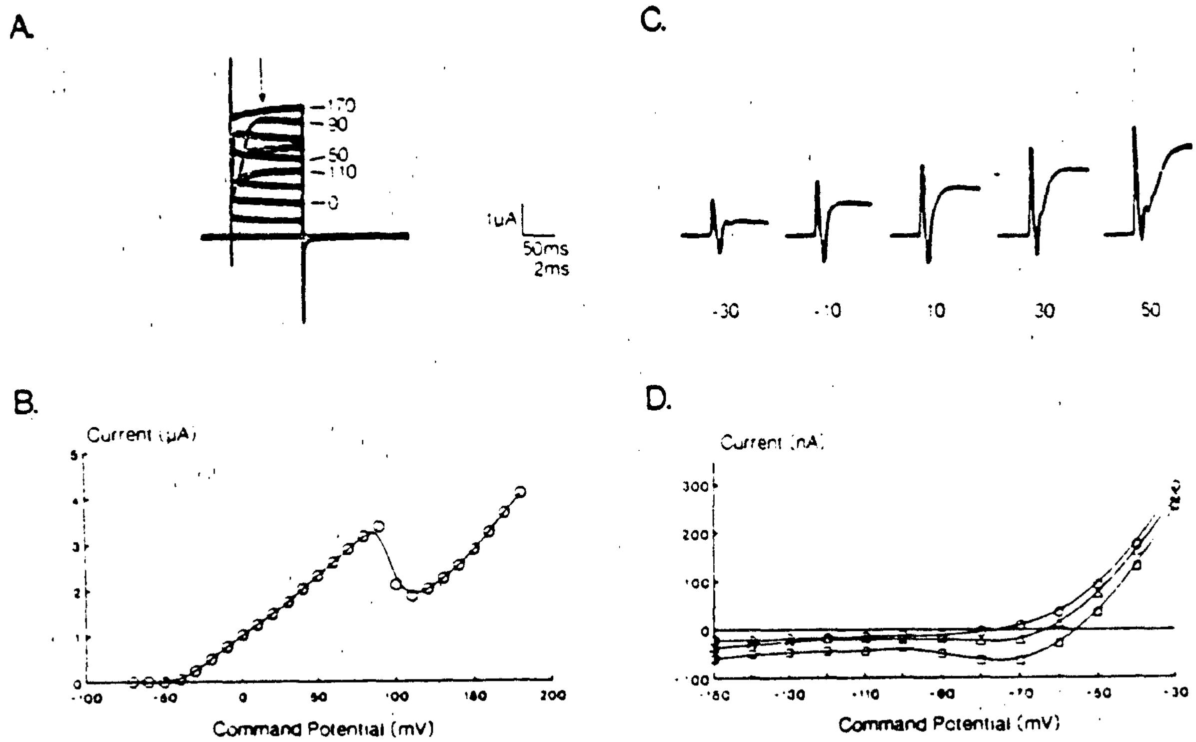


Fig. 1. A. Superimposed current responses to a range of command pulses (values indicated at right). Scale bar: 50ms. Arrow indicates the point at which current measurements were taken (50ms) to produce graph B. B. Typical N-shaped I-V curve. C. Current records showing the inward current component. Scale bar: 2ms. D. Dependence of tail currents on pre-pulse duration. 50mV pre-pulses were used; the holding potential was -70mV. Tail current measurements were made following pre-pulses of 10ms (circles), 30ms (triangles) and 50ms (squares). In this experiment, the polarity of tail currents reversed at -76mV, -64mV and -56mV for pre-pulses of 10, 30 and 50ms respectively.

REFERENCES

1. Cohen, M.J. & Jacklet, J.W. (1967). Phil. Trans. R. Soc. B 352:561-569.

2. Meech, R.W. & Standen, N.B. (1975). J. Physiol. (Lond.). 249:211-239.

3. Thomas, M.V. (1984). J. Physiol. (Lond.). 350:159-178.

4. Connor, J.A. & Stevens, C.F. (1971). J. Physiol. (Lond.). 213:21-30.

DIFFERENTIAL EFFECTS OF INORGANIC LEAD ON ION CHANNELS IN THE NEUROBLASTOMA CELL MEMBRANE.

Marga Oortgiesen, Regina G.D.M. van Kleef, Rajesh B. Bajnath and Henk P.M. Vijverberg.

Department of Veterinary Pharmacology, Pharmacy and Toxicology, University of Utrecht, P.O. Box 80.176, 3508 TD Utrecht, The Netherlands.

Much work has been carried out on the mechanisms underlying the neurotoxic effects of lead (Pb). It is assumed that the blocking effect of Pb on neuromuscular transmission is due to a competitive interaction between calcium (Ca) and Pb ions at extracellular as well as intracellular presynaptic sites[1]. Pb is supposed to affect neurotransmitter release by a blockade of the inward Ca current associated with nerve excitation and by a disturbance of the intracellular Ca balance. In the cultured neuroblastoma cell line N1E-115, effects of Pb on multiple ion channels can be studied separately. With the use of the whole-cell voltage clamp technique the effects of Pb on the nicotinic acetylcholine receptor and on two types of voltage-dependent Ca channels have been examined. In addition, a new intrinsic effect of Pb on the neuroblastoma cell membrane is described.

Pb causes a reduction of the amplitude of the ACh-induced inward current at very low concentrations. The concentration-effect relationship of the blocking action of Pb cannot be described by a sigmoidal curve. After exposure of the neuroblastoma cells to concentrations between 1 nM and 1 μM Pb, the degree of block increases from 25 % to 90 %. Higher Pb concentrations (≥ 10 μM) result in a reduction of this blocking action.

In N1E-115 cells a fast transient type I Ca current and a slower non-inactivating type II Ca current can be observed. Both types of Ca

1 and 10 μM Pb reduce the amplitude of both currents to half maximum values.

When the cell is superfused with high Pb concentrations (10-200 μM), a non-inactivating inward current is induced. The amplitude of this inward current depends on the Pb concentration. The nature of the Pb-induced current is not yet clear. It is likely that Pb exerts this effect by interacting with an external site, as internal application of the chelating agent EGTA does not affect the Pb-induced current and internal superfusion of the cell with 1 mM Pb failes to induce the same effect. The reversal potential of the Pb-induced current is similar to the equilibrium potential for sodium ions calculated from the Nernst equation. After replacement of all intracellular and extracellular sodium ions by potassium ions, no Pb-induced current could be measured. The Pb-induced current is not eliminated in the presence of external tetrodotoxin (TTX), tetraethylammonium (TEA), d-tubocurarine, atropine, and in chloride-free solutions. These results indicate that the Pb-induced current is mainly carried by sodium ions and that neither voltage-dependent sodium channels, potassium channels and chloride channels nor nicotinic and muscarinic ACh receptor-operated channels are involved. In outside-out membrane patches of neuroblastoma cells, application of 10 μM Pb causes the opening of ion channels with a single channel conductance of 13 pS.

From these results it can be concluded, that inorganic lead affects various ion channels of N1E-115 cells in a differential way. The nicotinic ACh receptor-mediated ion current appears to be more sensitive to low concentrations of Pb than the voltage-dependent Ca current. High Pb concentrations cause activation of ion channels that are permeable to sodium ions.

Manalis R.S., Cooper G.P., Pomeroy S.L. Brain Res. 1984, 294 95-109

ALLOSTERIC INTERACTIONS OF INSECTICIDES WITH THE [^{3}H]BTX-B BINDING SITE OF THE VOLTAGE-SENSITIVE SODIUM CHANNEL

Gregory T. Payne and David M. Soderlund

Department of Entomology, New York State Agricultural Experiment Station, Cornell University, Geneva, NY 14456 USA

Recent studies of the effects of DDT and pyrethroids on the activation of $^{22}Na^+$ uptake into mouse brain synaptosomes by veratridine, aconitine, and batrachotoxin demonstrate an insecticide-dependent increase in the potency of these compounds.[1] These findings implicate an allosteric effect of insecticides that increases the affinity of the activator recognition site. Binding interactions at the activator site can be detected using [^{3}H]batrachotoxinin A-20-α-benzoate (BTX-B), an analog of batrachotoxin.[2] In assays with rat brain synaptosomes, deltamethrin and the neurotoxic isomers of cypermethrin increased in affinity (K_D) of sodium channels for BTX-B without affecting the number of binding sites detected at saturation (B_{max}).[3]

We have initiated studies of the binding of [^{3}H]BTX-B to mouse brain preparations in an effort to define the mechanisms underlying insecticide-dependent enhancement of the potency of sodium channel activators. Equilibrium saturation studies of the binding of [^{3}H]BTX-B to mouse brain synaptoneurosomes[4] in the presence of saturating concentrations of *Leiurus quinquestratus* venom revealed a single class of sites having a K_D of 72 nM and a B_{max} of 7.1 pmol/mg protein. DDT and deltamethrin stimulated the specific binding of [^{3}H]BTX-B in a concentration-dependent manner. Equilibrium saturation studies of [^{3}H]BTX-B binding in the presence of a saturating concentration of DDT (100 μM) showed that this compound produced a two-fold increase in the affinity of sodium channels for [^{3}H]BTX-B but had no effect on binding capacity.

Preliminary studies have examined the effects of DDT on the association and dissociation kinetics of [^{3}H]BTX-B. DDT at 100 μM had no detectable effect on the initial rate of formation of the ligand-receptor complex but produced a two-fold increase in the half-life of this complex in dissocation experiments. The effects of DDT on dissociation are sufficient to account for the increased affinity of sodium channels for [^{3}H]BTX-B in the presence of DDT. This allosteric stabilization of the activator-sodium channel complex by insecticides is likely to be the principal mechanism underlying the increases in the potency of activators that were observed in sodium uptake experiments.

These findings demonstrate the utility of the [^{3}H]BTX-B binding assay to define the allosteric effects of insecticides on activator-sodium channel interactions. Moreover, this interaction permits the use of [^{3}H]BTX-B to explore indirectly the binding of insecticides to their recognition site on the the sodium channel.

1. Bloomquist, J. R. and Soderlund, D. M. Mol. Pharmacol. (submitted).
2. Catterall, W. A., Morrow, C. S., Daly, J. W. and Brown, G. B. J. Biol. Chem. 1981 **256** 8922-8927
3. Brown, G. B. and Olsen, R. W. Soc. Neurosci. Abst. 1984 **10** 865
4. Bloomquist, J. R. and Soderlund, D. M. Biochem. Biophys. Res. Commun. 1985 **133** 37-43

NEUROTRANSMITTER, NEUROMODULATOR AND Ca^{2+} CHANNEL LIGAND ACTIONS ON CULTURED RAT DRG NEURONES ARE REGULATED BY A PERTUSSIS TOXIN SENSITIVE G-PROTEIN.

Roderick H. Scott and Annette C. Dolphin
Department of Pharmacology, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE

Activation of a wide variety of receptors for neurotransmitters and neuromodulators ($GABA_B$, noradrenaline (α_2), dopamine, substance P, adenosine (A_1) and opiate) results in inhibition of neuronal Ca^{2+} channel currents. Guanine nucleotide binding proteins (G-proteins) appear to couple these receptors with Ca^{2+} channels. The $GABA_B$ receptor agonist(-)-baclofen (2-100μM) inhibited Ca^{2+} channel currents recorded from cultured dorsal root ganglion (DRG) neurones. This inhibitory action was prevented by pretreating the cells with pertussis toxin (2-5 hours 0.5μg/ml)[1]. These results suggest that a G_i or G_o type G-protein is involved in the response, as pertussis toxin selectively ADP-ribosylates α_i and α_o. Non-hydrolysable GTP analogues (GTP-γ-S, GMP-PNP) applied intracellularly using the whole cell recording technique inhibited the transient component of the Ca^{2+} channel current[2] and potentiated the action of (-)-baclofen[1]. The GDP analogue GDP-β-S applied intracellularly reduced the inhibitory actions of neurotransmitters and neuromodulators[1,3].

The residual Ca^{2+} channel currents recorded from cells containing internal GTP-γ-S had slowed rates of activation and were non-inactivating during a 100ms voltage step command[1]. The 1,4-dihydropyridine Bay K 8644 (5μM) which selectively increases L-type Ca^{2+} channel activity[4] potentiated Ca^{2+} channel currents by 26±8% and 107±37% (mean ± S.E.M., n=10) for control and GTP-γ-S contain cells respectively. Activation of a G-protein by GTP-γ-S appears to increase the sensitivity of cells to Bay K 8644 as well as resulting in selective inhibition of N and possibly T type Ca^{2+} channels.

Intracellular GTP-γ-S also induced marked changes in the actions of nifedipine (5μM), D_{600} (10μM) and diltiazem (30μM) three structurally different (1,4,-dihydropyridine, phenylalkylamine and benzothiazepine) Ca^{2+} channel ligands. In the absence of any GTP analogue these Ca^{2+} channel ligands inhibited Ca^{2+} channel currents[5], however, in the presence of internal GTP-γ-S they potentiated the current (1.5 to 4 fold)[6]. D_{600} decreased the time constant of activation of the peak Ca^{2+}

channel current (V_{clamp} = 0mV) recorded in the presence of GTP-γ-S from 9.5±2.3ms to 4.1±0.7ms (mean ± S.E.M. n=6). Internal GDP-β-S had no effect on the inhibitory actions of the Ca^{2+} channel ligands, but pre-incubation of the cells with pertussis toxin prevented the agonist effects of the ligands when GTP-γ-S was present inside the cells. The agonist actions of the Ca^{2+} channel ligands were voltage dependent[6,7] when the holding potential (V_H) was depolarized from V_H-80mV to V_H-30mV agonist effects were observed but were less pronounced[6]. The actions of D_{600} differ from those of nifedipine in that they are more transient and the agonist effect is not associated with a hyperpolarizing voltage shift in the current-voltage relationship, the mechanisms of regulation of Ca^{2+} channel ligand effects maybe different depending on the type of ligand.

We suggest that GTP-γ-S acting via a pertussis toxin sensitive G-protein interacts with different Ca^{2+} channel subtypes, inhibiting N-type channels and regulating the interactions of neurotransmitters, neuromodulators with both N and L channels, and Ca^{2+} channel ligands with L-type channels. Ca^{2+} channels appear to have multiple binding sites for each of the Ca^{2+} channel ligands[8], and the actions of the ligands are dependent on channel state (open, closed, inactivated). The inability of L-type channels to enter an inactivated state in the presence of GTP-γ-S activated G-protein may explain why antagonist actions of the Ca^{2+} channel ligands are prevented[6].

This work was supported by the MRC.

References

1. Dolphin, A.C. and Scott, R.H. J. Physiol, 1987. 386, 1-17.
2. Dolphin, A.C. and Scott, R.H. Trentham, D.R. and Wootton, J.F. J. Physiol, 1987. 386, 89P.
3. Holz, G.G., Rane, S.G. and Dunlap, K. Nature 1986. 319, 670-672.
4. Nowycky, M.C., Fox, A.P. and Tsien, R.W. Nature 1985. 316, 440-445..
5. Lee, K.S. and Tsien, R.W. Nature 1983, 302, 790-794.
6. Scott, R.H. and Dolphin, A.C. Nature 1987. 330, 760-762.
7. Brown, A.M., Kunze, D.L. and Yatani, A.J. J. Physiol. 1986 379, 495-514.
8. Striessnig, J., Goll, A., Mossburger, K. and Glossman, H. FEBS. 1986, 197, 204-210.

Block of Neuromuscular Transmission in Housefly Larvae by Cypermethrin; Electrophysiological and Ultrastructural Correlates.

Seabrook G.R., Duce I.R. and Irving S.N.

Dept. of Zoology, University of Nottingham, University Park, Nottingham, NG7 2RD U.K.

The effect of cypermethrin, an alpha-cyano pyrethroid, on the spontaneous and evoked release of neurotransmitter in late third instar housefly larvae was examined by intracellular, and extracellular patch recordings[1] from ventrolateral muscles 6a and 7a. All experiments were conducted at room temperature. Nerve terminal ultrastructure was examined using conventional transmission electron microscopy after fixation in 2.5 % glutaraldehyde and 1 % osmium tetroxide. The frequency of spontaneous miniature excitatory post-synaptic currents (mEPSCs; 0.276 + 0.326 hz, SD 35 cells) was potentiated (at least 10 fold) for several hours by treatment with 10 nM cypermethrin. This concentration of cypermethrin also blocked both neurally (early block) and electrotonically (late block) evoked responses. The amplitude of discrete synaptic currents (EPSCs), unlike excitatory post-synaptic potentials which were cumulative responses from at least 27 nerve terminals, were not depressed at the onset of early block (80.6 $\pm$ 23.9 pA c.f. control 81.0 $\pm$ 5.0 pA, SD 3 cells). However, the mean probability of quantal release declined with time after application of cypermethrin, with complete inhibition after ca. 20 minutes. This data is indicative of an all or none inhibition of neuromuscular transmission at discrete release sites, consistent with conduction block in the nerve terminal. Conduction block has also been implicated in deltamethrin and fenvalerate poisoning at this neuromuscular junction[2]. A 3 fold increase in the delayed release (DR) of transmitter (10-50 ms after a neurally evoked EPSC) immediately prior to early block suggests that calcium buffering following nerve terminal loading with this ion may have been suppressed; DR, like the quantal content of evoked responses, increases according to a high power function of $[Ca^{2+}]_o$[1]. Inhibition of mEPSC release in the later stages of cypermethrin poisoning was correlated with a decrease in the vesicular contents of nerve terminals (figure 1), unlike the near complete depletion of nerve terminals observed during deltamethrin poisoning[3]. A reduction in extracellular calcium ion concentration to 0.1 uM (buffered with EGTA) alleviated the depression in vesicular densities of motor nerve terminals treated with cypermethrin. In sections normal to the pre-synaptic dense body, electron lucent vesicles were observed to be highly localized around these "active zones" in both control and poisoned preparations (figure 1), although at the onset of neuromuscular block a greater number of vesicles were located immediately adjacent (<50 uM) to the active zone (31 $\pm$ 13 vesicles/ uM length of AZ; cf control 10 $\pm$ 4, SD 4 cells). This was correlated with the elevated frequency of spontaneous quantal discharges at this stage. In conclusion, the block of neuromuscular transmission by low concentrations of cypermethrin is consistent with a depolarizing phenomenon mediated by the block of conduction into the presynaptic element. The effect of cypermethrin on the densities and distributions of electron lucent vesicles within nerve terminals, and upon the frequency of quantal discharges does not conflict with a vesicular based theory of quantal neurotransmitter release.

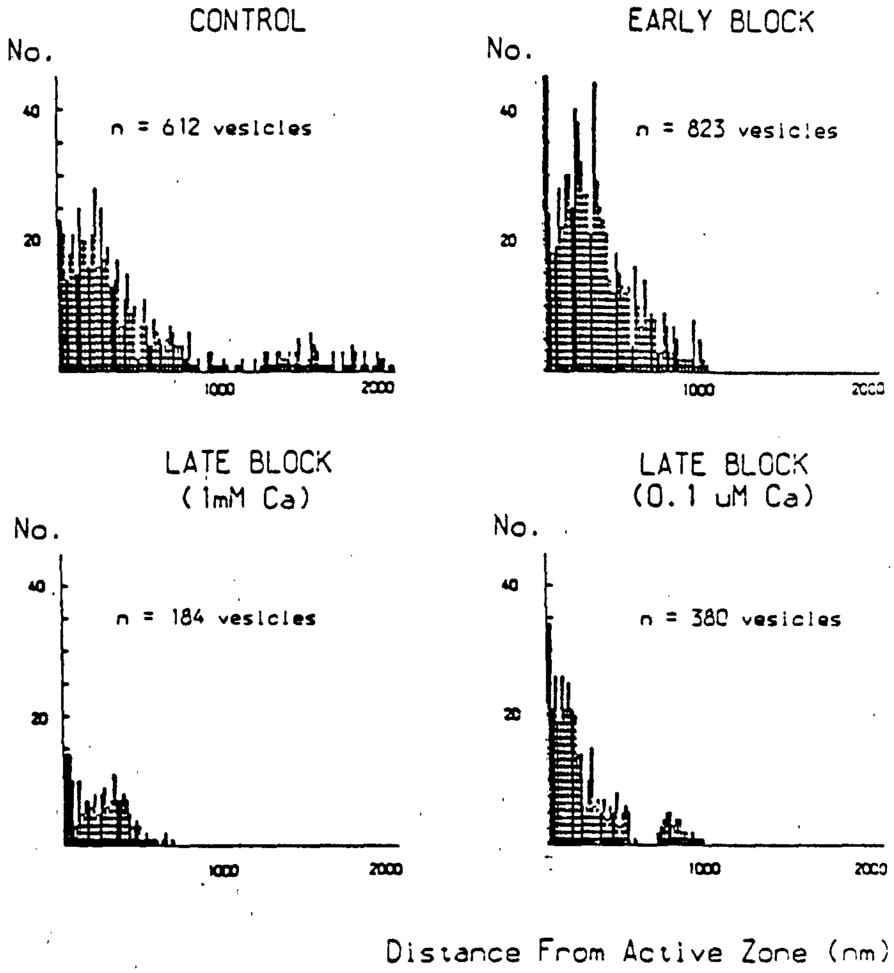
References

1. Seabrook G.R., Duce I.R., and Irving S.N. (1986) Soc. Neurosci. Abstr. 12(2); 819.

2. Salgado V.L., Irving S.N. and Miller T.A. (1983) Pestic. Biochem. and Physiol. 20; 169 - 182.

3. Schouest L.P., Salgado V.L. and Miller T.A. (1986) Pestic. Biochem. and Physiol. 25; 381 - 386.

FIGURE 1



Vesicle distribution relative to the presynaptic dense body was assessed only in sections normal to the active zone (N_T = 16 terminals), to ensure correct representation of vesicle organization around these structures. Treated preparations were fixed 15 minutes after application of 10 nM cypermethrin (early block), and 2 hours after application of 1 uM cypermethrin (late block).

Effects of the Pyrethroid Cypermethrin on L-glutamate Induced Changes in the Input Conductance of the Larval Housefly, Musca domestica, Ventrolateral Muscles.

Seabrook G.R., Duce I.R. and Irving S.N.

Dept. of Zoology, University of Nottingham, University Park, Nottingham, NG7 2RD U.K.

Cypermethrin has been demonstrated to block neuromuscular transmission in larval housefly ventrolateral muscles (VLMs)[1,2]. Although the effect is consistent with a presynaptic depolarization, as it involves an increase in miniature frequency and decrease in the amplitude of evoked excitatory potentials, its postsynaptic action(s) have not yet been assessed. Thus, this study was undertaken to observe the effects of both cypermethrin and its solvent acetone upon the postsynaptic aspect of synaptic transmission. VLMs 6a and 7a had similar resting membrane potentials (57.6 ± 8.0 and 59.2 ± 8.3 respectively, n = 20 cells) and input conductances (3.08 ± 0.87 and 2.97 ± 1.00 respectively, n = 12 cells). L-glutamate produced a dose-dependent depolarization and increase in the sarcolemma input conductance (with no distinct maximum at up to 10 mM L-glutamate). A Hill plot revealed that a minimum of 1 glutamate molecule was required to bind to the receptor to elicit a unit increase in input conductance. At concentrations of 0.1% acetone and 10 nM cypermethrin, no effects were observed on the resting muscle membrane potential, the sarcolemma input conductance, or sensitivity to 10 mM L-glutamate (pretreated with 1 uM concanavalin A to block glutamate receptor desensitization). However, at higher concentrations both acetone and cypermethrin were observed to have postsynaptic effects. At 1% acetone caused a subtle increase in muscle input resistance ($P < 0.05$), although higher concentrations produced a marked decrease in input resistance (EC_{50} at 4%), and a membrane depolarization upon its initial application which was followed by a sustained membrane hyperpolarization. Extensive axonal repetitive firing was also noted with acetone concentrations above 1%. 1 uM cypermethrin, 30 - 90 minutes after application, blocked the L-glutamate induced increases in input conductance (table 1), as well as inducing substantial oscillations in the resting sarcolemma membrane potential. The initial membrane depolarizations induced by 10 mM L-glutamate in control preparations were still observed in muscles poisoned with 1 uM cypermethrin. These data demonstrate that the block of neuromuscular transmission at larval housefly nerve terminals by 10 nM cypermethrin does not involve a change in muscle membrane input conductance or a decrease in the sarcolemma sensitivity to L-glutamate, and therefore is likely to be a consequence of its presynaptic action.

References

1. Seabrook G.R., Duce I.R., and Irving S.N. (1988) Quantal Neurotransmitter Release and Pyrethroid Insecticide Action on the Larval Housefly (Musca domestica) Neuromuscular Junction. Pesticide Science, in press.

2. Seabrook G.R., Duce I.R., and Irving S.N. Block of Neuromuscular Transmission in Housefly Larvae by Cypermethrin; Electrophysiological and Ultrastructural Correlates. Neurotox 88.

TABLE 1

	Control	Cypermethrin 10 nM	Cypermethrin 1 uM
Resting Input Conductance	3.64 ± 1.05	3.99 ± 1.53	3.16 ± 0.50
Initial effect of 10 mM L-glu	4.92 ± 1.62*	5.53 ± 2.45*	3.24 ± 0.43#
Initial Depolarization (mV)	8.9 ± 2.0	14.6 ± 4.9	12.5 ± 3.8
Stabilized effect of 10 mM L-glu	4.64 ± 1.43*	5.43 ± 1.87*	3.23 ± 0.50#
Recovery after wash off	3.65 ± 1.00	4.01 ± 1.44	3.26 ± 0.73
% Recovery	101 ± 5	101 ± 4	103 ± 10
Net Change in Input Conductance	1.03 ± 0.85	1.44 ± 0.45	0.07 ± 0.30

Means ± SD from 7 cells for each treatment. Cells were pretreated with 1 uM con A for 30 minutes. Conductance measurements (x 10^{-7} Siemens) were made after a further 30 minutes equilibration in cypermethrin, or normal saline. Stabilized responses were measured 2 minutes after application of L-glutamate (with the appropriate concentration of cypermethrin where necessary). * significantly different at 0.01 level from resting input conductance, # not significantly different at 0.05 level (2 way analysis of variance).

BLOCK OF AN INSECT CNS GABA RECEPTOR BY CYCLODIENE AND CYCLOHEXANE INSECTICIDES

Keith A. Wafford, Sarah C.R. Lummis, and David B. Sattelle.

Department of Zoology, University of Cambridge, Cambridge CB2 3EJ

The cyclodiene and cyclohexane insecticides are chlorinated hydrocarbons, whose symptoms of poisoning are convulsive seizures and increased spontaneous neuronal activity. Recently these compounds have demonstrated an ability to displace the binding of [^{3}H]TBPS, a ligand for the GABA-regulated chloride channel[1], and inhibit GABA-induced $^{36}Cl^-$ influx into rat brain membrane microsacs[2,3]. In insects, cyclodienes will inhibit chloride uptake into cockroach muscle[4], but there has been no direct physiological demonstration of a blocking action on insect GABA receptors. The fast coxal depressor motor neurone in the cockroach metathoracic ganglion is known to possess GABA receptors[5], and CNS homogenates will demonstrate GABA-activated $^{36}Cl^-$ influx[6].

Endrin (a cyclodiene) and lindane (a cyclohexane) showed a dose dependent block of the GABA response on motor neurone D_f, with IC_{50}'s of 5.0×10^{-7}M and 5.0×10^{-6}M respectively. The block tended to flatten the GABA dose-response curve (Figure 1a,b) and could not be overcome with higher doses of GABA, suggesting a non-competitive type of inhibition. At higher concentrations, endrin demonstrated direct effects on the cell body membrane, at 1.0×10^{-4}M endrin caused an irreversibly hyperpolarization of the membrane together with a decrease in conductance. Endrin block was also dependent on the direction of chloride ion flow, being much less potent at blocking depolarizing GABA responses,

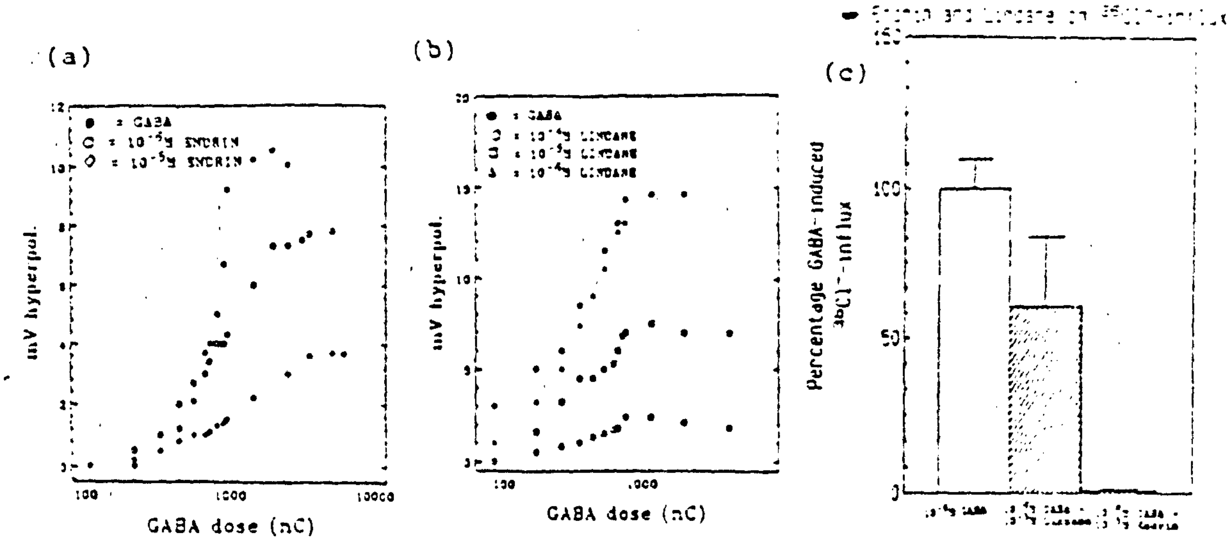


Figure 1. Effects of (a) endrin at 1.0 x 10^{-6}M and 1.0 x 10^{-5}M and (b) lindane at 1.0 x 10^{-6}M, 1.0 x 10^{-5}M, and 1.0 x 10^{-4}M, on the GABA dose-response curve of motor neurone D_f with membrane potential held at -60mV, and (c) 1.0 x 10^{-5}M endrin and lindane on the GABA-activated chloride-influx into the cockroach CNS microsac preparation.

when intracellular chloride was elevated. These insecticides were also ineffective at blocking chloride-mediated glutamate responses on the same cell. Lindane and endrin were also found to inhibit GABA-mediated $^{36}Cl^-$ influx into crude microsacs from cockroach CNS tissue, endrin being more potent than lindane (Figure 1c). These findings suggest that endrin and lindane inhibit GABA receptor function in a similar fashion to picrotoxin, with IC_{50}'s analogous to that seen with vertebrate binding and chloride flux assays.

1. Lawrence L.J. and Casida J.E. (1984) Life Sci., 35 171-178.
2. Abalis I.M., Eldefrawi M.E. and Eldefrawi A.T. (1986) J. Toxic. and Env. Health, 18 13-23.
3. Gant D.B., Eldefrawi M.E. and Eldefrawi A.T. (1987) Toxicol. Appl. Pharmac. 88 313-321.
4. Ghiasuddin S.M. and Matsumura F. (1982) Comp. Biochem. Physiol. 73C 141-144.
5. Sattelle D.B., Pinnock R.D., Wafford K.A. and David J.A. (1988) Proc. Roy. Soc. Lond. B In Press.
6. Wafford K.A., Sattelle D.B., Abalis I.M., Eldefrawi A.T. and Eldefrawi M.E. (1986) J. Neurochem. 48 177-180.

Effect of taurine on $^{45}Ca^{2+}$ uptake and acetyl-choline release in locust synaptosomes

Peter S. Whitton[1], Russell A. Nicholson[2] and Robin H.C. Strang[1]

1 Department of Biochemistry, University of Glasgow, Glasgow, G12 8QQ
2 Wellcome Research Laboratories, Ravens Lane, Berkhamsted, Hertfordshire HP4 2DY

INTRODUCTION

Taurine (2-aminoethanesulphonic acid) is an abundant amino acid found in both lower and higher organisms, (1). Taurine occurs in the insect nervous system, (2) sometimes at very high concentrations, (3). In recent years evidence has accumulated that taurine may function as a neuromodulator in vertebrates, possibly by modifying calcium homeostasis, (4). We have recently been studying the effects of taurine on the nervous system of Schistocerca americana gregaria, and have evidence that taurine may function as a neuromodulator in insects also.

EXPERIMENTAL

Experiments were performed using a crude synaptosomal preparation, obtained as previously described, (5). Synaptosomes were incubated and perfused in insect saline (mM:NaCl 214; KCl, 3.1; $CaCl_2$ $6H_2O$, 2.05; Na_2HPO_4. $12H_2O$/ NaH_2PO_4 $2H_2O$ 0.2; M Cl_2. $6H_2O$ 6.6; glucose, 16.7). ^{45}Calcium uptake was estimated using a 2 min. incubation period (about 100 µg synaptosomal protein with 2 uCi $^{45}Ca^{2+}$ in 200 ul saline) which was terminated by rapid filtration through Millipore Filters (0.45 um pore size).

^{3}(H) ACh release was studied by perfusing synaptosomes which had been reincubated with 12.5 µCi ^{3}(H) choline and were then loaded onto Whatman GF/B filters (about 90 µg protein per filter).

Synaptosomes were depolarised using either Veratridine (100 µm) or high $[K^+]$ (100 mM).

RESULTS AND DISCUSSION

Depolarised synaptosomes were found to accumulate considerably more $^{45}Ca^{2+}$ than those in the resting state. When synaptosomes were depolarised using either Veratridine or high $[K^+]$, taurine caused a concentration-dependent decrease in $^{45}Ca^{2+}$ uptake (Fig.1). Both depolarising agents caused release of (^{3}H) ACh, and this was found to be largely, but not entirely, dependent on the presence of external calcium (data not shown). Taurine was observed to cause a concentration-dependent decrease in (^{3}H) ACh release which was significant at all taurine concentrations used. The reduction in (^{3}H) ACh release by taurine was proportionally less than the decrease on $^{45}Ca^{2+}$ uptake (Fig.1), and may reflect the fact the ACh release was not found to be wholly calcium-dependent in the present system. Taurine has been observed to reduce (^{3}H) ACH release from a number of mammalian preparations (6,7), and such findings have identified taurine as a possible neuromodulator in mammals. The present results suggest that taurine may serve a similar role in insects.

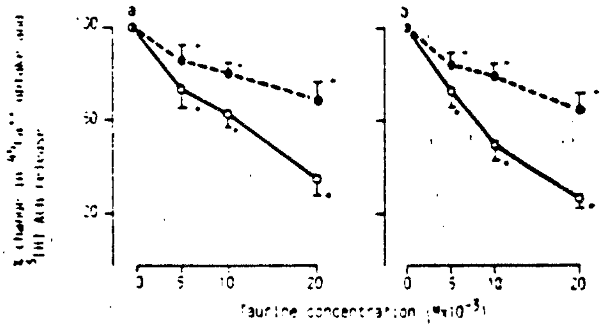


Figure 1. Effect of different concentrations of taurine on Ca^{++} uptake(o), and ^{3}(H) ACh(o) release from crude synaptosomal preparation from the locust <u>S.gregaria</u>, depolarised by (a) 100mM K^+, or (b) 100uM Veratridine. In both cases controls free of taurine are taken as 100%. Results significantly different from controls are indicated thus: +P 0.05 *P 0.01.

REFERENCES

1. Jacobsen J.C. and Smith L.H. Physiol Rev. 1968. <u>48</u>. 424-511.
2. Whitton P.S., Nicholson R.A. and Strang R.H.C. Trans. Biochem. Soc. 1986, <u>22</u> 91-92.
3. Osborne, N.N. Comp. Gen. Pharmacol. 1971, <u>2</u>, 433-438.
4. Pasantes-Morales H. and Gamboa A. J. Neurochem. 1980, <u>34</u>, 244-246.
5. Whitton P.S., Nicholson R.A. and Strang R.H.C., Insect Biochem. 1987, <u>17</u> 573-577.
6. Kuriyama W., Muramatsu M., Nakawaga K. and Wakita K. In 'Taurine and Neurological Disorders' 1978, pp 201-216 (Huxtable R.T. and Barbeau A. Eds.) Raven Press, New York.

THE POND SNAIL BRAIN: A MODEL SYSTEM FOR CELLULAR STUDIES ON GENERAL ANAESTHETICS.

William Winlow

Department of Physiology, University of Leeds, Leeds LS2 9NQ

In spite of their clinical importance the cellular actions of general anaesthetics are poorly understood. Recently the pond sail, Lymnaea stagnalis has proved to be an excellent model system for the study of the modes of action of general anaesthetics, since behavioural, cell physiological and biophysical experiments can all be performed on this preparation (1,2)

Intact specimens of Lymnaea are anaesthetised by near clinical doses of the inahalational anaesthetics halothane (ED50: 0.83% v/v), enflurane (ED50: 1.01% v/v) and isoflurane (ED50: 1.09% v/v). Concentrations of halothane between 0.5 and 2.0% (v/v) produce dose-dependent effects on the spontaneous discharge of a number of cells. Low doses (0.5 to 1.0%) cause patterning of spontaneous discharges and weak bursting, whilst at higher doses spike frequency declines and all cells eventually become quiescent. However a comparison of the effects of several different inhalational and systemic general anaesthetics (halothane, enflurane, ketamine, thiopentone, pentobarbitone) reveals important differences in the responses of individual cell types to applied anaesthetics, irrespective of the anaesthetic used (3). Depending on the type of neurone studied there is either a gradual tendency towards quiescence or the occurrence of paroxysmal depolarising shifts and oscillatory behaviour during both spontaneous and evoked activity (Figure 1). Clearly, different neuronal

C. BIOCHEMISTRY AND MOLECULAR BIOLOGY OF NEUROTRANSMISSION

membranes respond to anaesthetic agents in different ways. This finding may have important implications for our understanding of the differential actions of anaesthetics on different regions of the vertebrate brain.

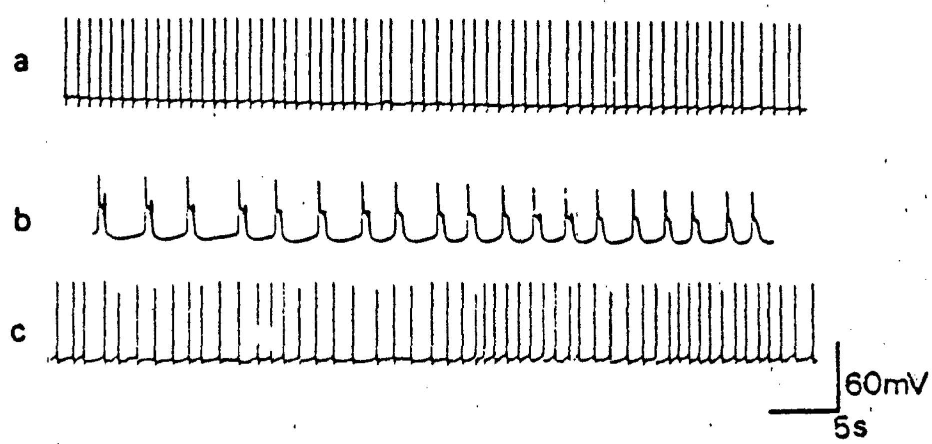


Figure 1 Different types of neurone take different pathways to silence independent of the anaesthetic used. In many neurones there is a gradual decline of discharge frequency whilst other neurones, such as the one shown here, develop a series of paroxysmal depolarising shifts of membrane potential prior to quiescence. a) Pre-control in normal saline ; b) after 6 minutes in saline containing 0.2 mM Ketamine; c) post-control, 30 minutes after rinsing in fresh saline.

1. Winlow, W., Girdlestone, D., Cruickshank, S.G.H. McCrohan C.R. In Neurobiology: Molluscan Models (eds. H.H. Boer et al) 1987. 132-137. North-Holland Publishing Co., Amsterdam.
2. Girdlestone, D., Cruickshank, S.G.H., Winlow, W. Comp. Biochem. Physiol. C. 1988 (in press)
3. Girdlestone, D. & Winlow, W. J. Physiol. 1987 386 33P

PURIFICATION AND CHARACTERIZATION OF MOSQUITO ACETYLCHOLINESTERASE.

Bryony C. Bonning

Department of Entomology, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT

A purification protocol has been developed for mosquito acetylcholinesterase (AChE). The detergent Triton X-100 was used for enzyme solubilization, prior to centrifugation and procainamide affinity chromatography. A 350 fold purification was achieved.

Purified AChE was characterized by sucrose gradient centrifugation in the presence of detergent, and polyacrylamide gradient gel electrophoresis under various conditions. The purified AChE exhibited the same sedimentation coefficient, molecular weight and hydrodynamic properties as crude AChE.

Sucrose gradient centrifugation in the presence of 1% Triton X-100 gave a single peak with a sedimentation coefficient of 9. The enzyme behaved heterogeneously in the absence of detergent. On polyacrylamide gel electrophoresis in the presence of Triton X-100, a 135 kDa electromorph was reduced by 2-mercaptoethanol, or protease treatment to a 67kDa form. A third form showing a high, somewhat variable molecular weight in the gels is markedly hydrophobic, as shown by enhanced migration in gels containing sodium deoxycholate.

I propose that mosquito AChE exists as a hydrophobic form, possibly an aggregate, reducable to relatively hydrophilic dimeric and monomeric forms.

AFFINITY LABELLING OF OPIOID RECEPTORS.

Anna Borsodi, Sándor Benyhe, Éva Varga, József Simon and Mária Wollemann.

Institute of Biochemistry Biological Research Center of Hungarian Academy of Sciences 6701 Szeged, P.O.Box 521 Hungary

We have recently synthesized a number of affinity ligands[1-6] in normal and in tritiated forms which are able to react irreversibly at opioid receptors. Chloromethyl ketone derivatives of leucine enkephalin, D-Ala^2-Leu^5-enkephalin, D-Ala^2-D-Leu^5-enkephalin and oxymorphazone (14-OH-dihydromorphine) exhibit high affinity for opioid receptors in rat and frog brain. Preincubation of the membrane fractions with these ligands causes a significant inhibition of ^{3}H-naloxone binding which cannot be reversed by extensive washing. Affinity of enkephalin chlormethyl ketones and oxymorphazone toward the mu sites is much greater than for the delta sites. It was also found, that the irreversible inhibition is selective for the high affinity (K_d 1 nM) ^{3}H-naloxone binding site (putative mu - 1 - site).

We conclude, that these derivatives can be used as affinity labels for the opioid receptors, allowing us to study the structure of the mu receptor subtype.

1. Szűcs, M., Benyhe, S., Borsodi, A., Wollemann, M., Jancsó, G. Szécsi, J. and Medzihradszky, K. Life Sci. 1983 32 2777-2784

2. Benyhe, S., Hepp, J., Szűcs, M., Simon, J., Borsodi, A., Medzihradszky, K. and Wollemann, M. Neuropeptides 1986 8 173-181

3. Tóth, G., Hepp, J., Sirokmán, F., Medzihradszky, K. J. of Labelled Comp. and Radiopharmaceuticals XXIV 1987 859-866

4. Szűcs, M., Simon, J., Belcheva, M., Benyhe, S., Borsodi, A., Hepp, J., Medzihradszky, K., Tóth, G. and Wollemann, M. J. Neurochem. 1985 44 Suppl. S87D

5. Newman, E.L., Borsodi, A., Tóth, G., Hepp, J. and Barnard, E.A. Neuropeptides 1986 8 305-315

6. Varga, E., Tóth, G., Benyhe, S., Hosztafi, S. and Borsodi, A. Life Sci. 1987 40 1579-1588

Investigation of Cloride Flux in Membrane Vesicles Prepared from the Supraoesphogeal Ganglia of Locust
Schistocerca gregaria.

M.C.S. Brown, G.G. Lunt & A. Stapleton*. Department of Biochemistry, University of Bath, Bath, BA2 7AY. *Insecticide Discovery Laboratory, Dow Chemical Company, Walnut Creek, CA94598, U.S.A.

We have previously investigated the binding of the cage convulsant, [^{35}S]-t-butylbicyclophosphorothionate (TBPS) to membranes prepared from the supraoesphogeal ganglia of locust (Brown *et. al.* 1987). This binding was insensitive to the convulsant picrotoxinin (Ptx), which is a competitive inhibitor of TBPS binding to mammalian $GABA_D$ receptors. (Squires *et. al.* 1983). However we have also shown in the same membranes that Ptx could reduce the GABA enhancement of benzodiazepine binding (Rutherford *et. al.* 1987). Thus we deduce that locust ganglion membranes do contain a binding site for Ptx but that its relationship to the TBPS binding site, is different from that in mammalian brain. We are confident that the TBPS is binding to a GABA receptor complex similar to the $GABA_A$ complex in mammals, as the binding is affected by GABA, barbiturates and benzodiazepines.

The purpose of this study was to investigate how the TBPS and Ptx binding sites related to the chloride channel, which they are both supposed to interact with (Squires *et. al.* 1983). To do this we have set up a chloride flux assay in membrane vesicles (microsacs) from the same ganglia of the locust. These microsacs were prepared by a very gentle homogenisation, and no further preparation steps, except the filtration of the homogenate through nylon boulting cloth of 159 μM pore size. Initial experiments have shown that we can measure GABA stimulated chloride flux which has an effective concentration, 50% of 7 μM. This is in reasonable agreement with chloride flux data from Abalis *et al.* (1986) in rat brain microsacs and Ghiasuddin & Matsumura (1982) in cockroach nerve cords. However the time course for uptake is more in agreement with those workers who have used microsac preparations, from rat brain (Abalis *et al.* 1986) and cockroach nerve cords (Wafford *et al.* 1987), reaching a maximum within 30 seconds. The GABA stimulated chloride flux was blocked by 4,4'-diisothiocyano-2,2'-stilbenedisulphonic acid (DIDS) a chloride channel blocker (White & Miller, 1979) and Ptx.

Abalis, I.M., Eldefrawi, M.E. & Eldefrawi, A.T. Effects of insecticides on GABA induced chloride influx into rat brain microsacs. J. Tox. & Environmental Health 18 (1986) 13-23.

Brown, M.C.S., Lunt, G.G. & Stapleton, A. Further characterisation of the [^{35}S]-TBPS binding site in locust ganglia membranes. Comp. Biochem. Physiol. (in press).

Ghiasuddin, S.M. & Matsumura, F. Inhibition of gamma-aminobutyric acid (GABA) induced chloride uptake by gamma-BHC and heptachlor epoxide. Comp. Biochem. Physiol. 73C (1982) 141-144.

Rutherford, D.M., Jeffries, D., Lunt, G.G. & Weitzman, P.D.J. GABA binding to receptor sites in locust supraoesphogeal ganglia. Neurosci. Letters 79 (1987) 337-340.

Squires, R.F. Casida, J.E., Richardson, M. & Saederup, E. [^{35}S]-TBPS binds with high affinity to brain-specific sites coupled to GABA and ion recognition sites. Mol. Pharmacol. 23 (1983) 326-336.

Wafford, K.A., Sattelle, D.B., Abalis, I., Eldefrawi, A.T. & Eldefrawi, M.E. gamma-Aminobutyric acid(GABA) -activated $^{36}Cl^-$ Influx: A functional in vitro assay for CNS GABA receptors of insects. J. Neurochem. 48 (1987) 177-180.

White, M.M. & Miller, C. A voltage-gated anion channel from the electric organ of *Torpedo californica*. J. Biol. Chem. 254 (1979) 10161-10166.

Immunocytochemical Localisation of L-Glutamate in Motor Innervation of Locust (Schistocerca gregaria) Skeletal Muscle.

Timothy C. Budd and Ian R. Duce.

Department of Zoology, University of Nottingham, University Park, Nottingham NG7 2RD, England.

There is considerable evidence to suggest that L-glutamate (L-glu) is the neurotransmitter at excitatory motor synapses in insects[1]. However direct evidence for the presence of L-glu in nerve terminals is less convincing. Recently the localisation of amino acid neurotransmitters in the mammalian CNS has become possible using specific antisera[2]. We report here the application of this technique to the locust retractor unguis muscle using a polyclonal antiserum raised in our laboratory against L-glu conjugated with glutaraldehyde (G) to bovine serum albumin (BSA), haemoglobin (Hb) and poly-l-lysine (PL).

A sequential injection regime of these conjugates [3] was employed to raise an antiserum in rabbits. The antiserum was further purified using affinity chromatography on Sepharose-BSA-G and Sepharose-BSA-G-L-glu columns and tested for specificity against a range of amino acids. Cross-reactivity was examined by spotting a range of amino acids onto activated nitrocellulose[4] and visualising immunoreactivity using swine anti-rabbit serum and peroxidase anti-peroxidase. The antiserum reacted specifically with L-glu but not with Gaba, L-aspartate, D-aspartate, D-glutamate, L-serine, L-glutamine, L-threonine, L-alanine, L-proline, L-leucine, L-glycine, quisqualic acid or kainic acid.

Retractor unguis muscles were prepared for immunocytochemistry by fixation in 1% G and 1% formaldehyde. To investigate the presence of an active uptake system for L-glutamate some muscles were incubated in 0.5 uM L-glutamate prior to fixation. For light and electron microscopy the unlabelled antibody/PAP method was used. Muscles were examined by light microscopy as wholemounts cleared in methyl salicylate and for electron microscopy the preparations were postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Spurr resin prior to sectioning. L-glutamate-like immunoreactivity was also visualised under the scanning electron microscope using 0.5um latex beads coated with swine anti-rabbit serum[6] to identify binding of anti-L-glutamate serum.

Light microscopy revealed dense immunoreactivity in superficial nerves on the surface of the retractor unguis muscle. The use of affinity purified antiserum produced a more specific pattern of staining as compared with muscles treated with the whole antiserum. Electron microscope immunocytochemistry using purified antiserum confirmed these findings and showed that immunoreactivity was predominately a feature of nerve terminals and preterminal axoplasm. In preparations which had been pretreated with L-glutamate the amount of immunoreactivity in the

nerve terminals was increased and there was also appreciable levels of staining in nerve terminal glia and associated wuth the post-synaptic region. Scanning electron microscopy provided an additional method for visualising the immunoreactivity, and clumps of latex beads were found overlying the surface of nerve terminals.

(This work was supported by SERC project grant GR/E 43638).

1. Piek T. Comprehensive Insect Physiology Biochemistry and Pharmacology 11 1985.

2. Ottersen, O.P. Storm-Mathisen, J. TINS. 10 250-255 1987.

3. Sequela, P. Geffard, M. Buijs, R.M. le Moal, M. PNAS. 81 3888-3892 1984.

4. Hodgson, A.J. Penke, B. Erdei, A. Chubb, I.W. Somogyi, P. J.Histochem. Cytochem. 33 229-239 1985.

5. Ottersen, O.P. Storm-Mathisen, J. Neurosci. 16 589-606 1985.

6. Schriber, R.A., Zucker-Franklin, D. Cell Immunol.14 470-474 1974.

BINDING OF SAXITOXIN TO VOLTAGE DEPENDENT SODIUM CHANNEL OF INSECT NERVE MEMBRANE PREPARATION

Ash. K. Dwivedy

AFRC Unit, Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ.

Saxitoxin (STX) and tetrodotoxin (TTX) specifically inhibit sodium current in many excitable membranes, and a highly selective action of these toxins at nanomolar concentration has led to their extensive use for the determination of the sodium channel receptor density in variety of tissues[1]. Synthetic STX, STX and TTX of natural origin were used in voltage clamp studies to determine STX as a potent specific inhibitor for sodium channels on isolated giant axons of the cockroach, Periplaneta americana[2]. Sodium transport dependent on voltage sensitive sodium channels in cockroach central nervous system (CNS) synaptosomes was specifically inhibited by TTX ($K_{0.5}$ equivalent to 11 nM), and its efficacy was qualitatively distinct than that compared with vertebrate synaptosomes, membrane vesicles and cultured neuron[3,4]. The present study describe the binding of [^{3}H]STX under the physiological conditions which are able to inhibit ^{22}Na transport and alter membrane potential in the synaptosomes prepared from the CNS of P. americana. Using a standard rapid filteration assay, the saturable components of voltage sensitive sodium channel was analysed in various nerve membrane fractions prepared after centrifugation procedure[5].

The result suggest that STX blocks the ^{22}Na influx through veratridine-activated Na^+ channels with a K_i (6×10^{-9}M) that is similar to the K_d (3.6×10^{-9}M) observed for STX binding to synaptosomes under the similar conditions. The association rate constant ($K_1 = 2.3 \times 10^{8}\ M^{-1}S^{-1}$) and dissociation rate constant ($K_{-1} = 7.6 \times 10^{-1}S^{-1}$), yielded a K_d of 3.31 $\times 10^{-9}$ M. The concentration of competing unlabelled compounds which

displaced the binding of $[^3H]STX$ by 50% at equilibrium conditions was for STX ($K_{0.5} = 6 \times 10^{-9}M$) and for TTX ($K_{0.5} = 8 \times 10^{-8}M$). Linear regression of Hill plots of STX binding to synaptosomes gave Hill coefficients ranging from 0.8 to 1.0. A reasonable description of these results suggest to a single site competition model in insect synaptosomes membranes. With this model a simple relation exists between the free competitor concentration at half displacement, $K_{0.5}$ and the K_d of the competitor.

$$K_d = K_{0.5} / [1 + ([^3H]STX]_{0.5} / K_d STX)] \qquad (1)$$

where K_d STX is K_d for STX binding and $[^3H]STX_{0.5}$ is free concentrations of $[^3H]STX$ at half maximal displacement. By competitive displacement analysis, the K_d values for TTX were $2.9 \times 10^{-8}M$ for insect CNS synaptosomes as derived by application of equation (1) to the binding data. The results confirm previous reports that the relative affinity of these toxins in insects nerve membrane is STX>TTX as measured by action potential[2], though are in contrast to the pharmacological results reported in <u>Musca domestica</u> head extracts[6].

REFERENCES

1. Ritchie, J.M. and Rogart, R.B. Rev. Physiol. Biochem. Pharmacol. 1977, <u>79</u>, 1-50.

2. Sattelle, D.B., Pelhate, M., and Hue, B. J. exp. Biol. 1979, <u>83</u>, 41-49.

3. Dwivedy, A.K. In Insect Neurochemistry and Neurophysiology (edited by Borkovec A.B. and Gelman D.B.) The Humana Press, 1986, 393-396.

4. Dwivedy, A.K. Biochem. Soc. Trans. 1987, <u>15</u>, 145-146.

5. Dwivedy, A.K. Pestic. Sci. 1985, <u>16</u>, 615-626.

6. Pauron, D., Barhanin, J. and Lazdunski, M. Biochem. Biophys. Res. Commun. 1985, <u>131</u>, 1226-1233.

[illegible] GLUTAMATE, QUISQUALATE AND GABA RECEPTORS IN XENOPUS LAEVIS OOCYTES BY INJECTION OF mRNA FROM LOCUST LEG MUSCLE

[illegible][*], Mustafa B.A. Djamgoz[*], Peter N.R. Usherwood[‡], George [illegible][‡], Jeannette O'Brien[+], Mark G. Darlison[+] and Eric A. Barnard[+].

[*]Department of Pure and Applied Biology, Imperial College of Science and Technology, London, SW7 2AZ; [‡]Department of Zoology, University of Nottingham, Nottingham NG7 2RD; MRC Molecular Neurobiology Unit, MRC Centre, Hills Road, Cambridge CB2 2QH.

Several groups have shown that injection of messenger ribonucleic acid (mRNA) from various sources into Xenopus oocytes can lead to expression of membraneous proteins present in the tissue of origin.[1,2] Using this technique we have demonstrated that mRNA extracted from locust skeletal muscle can induce transmitter receptors similar to those which have been found in the intact muscle.[3,4,5]

poly(A)$^+$ mRNA was extracted from leg muscles of adult locusts (Schistocerca gregaria), as described previously.[6] 40-60 nl of aqueous mRNA at a concentration of 1 ug/ul was injected into each oocyte. The oocytes were then stored in a modified Barths medium[7] at 20°C until use. During electrophysiological recordings, oocytes were perfused at 2 ml/min with frog Ringer (NaCl 115 mM; KCl 2.5 mM; $CaCl_2$ 1.8 mM; HEPES 10mM; pH 7.2). Two-electrode voltage-clamp was used and drugs were applied in bath, mostly at a holding potential of -60 mV. The set-up allowed up to five different solution changes to be performed on a given oocyte.

Expression of glutamate, quisqualate and GABA receptors occurred 5 to 9 days after injection of the mRNA. The results obtained are summarised in Table 1 (n=number of experiments, each from a different oocyte).

Table 1 : Some electrophysiological characteristics of the neurotransmitter receptors expressed in Xenopus oocytes by mRNA from locust leg muscle.

RECEPTOR TYPE	REVERSAL POTENTIAL (mV)	TYPE OF CURRENT	POSSIBLE ION(S) INVOLVED
GLUTAMATE	-61 ± 17 (n=8)	smooth	$Na^+/K^+/Cl^-$ (?)
QUISQUALATE	-10 ± 15 (n=10)	generally smooth; occasional oscillations	$Na^+/K^+/Ca^{2+}$ (?)
GABA	-24 ± 2 (n=12)	smooth	Cl^-

Agonist-induced currents generally had peak values of 50 nA, and in the

case of glutamate and quisqualate desensitisation often occured. Desensitisation could not be reversed even by prolonged washing with normal Ringer. However, by 15 minute pre-treatment of oocytes with 1uM concanavalin A, desensitisation could often be prevented. All effects were dose-dependent in the concentration range 1 uM to 1 mM. It was also noted that individual oocytes did not necessarily express all three receptors at the same time.

Application of 0.1 mM GABA onto oocytes clamped at -60mV produced inward currents of up to 50 nA. The reversal potential of this current was highly consistent and clearly suggests that it is carried by chloride ions. This is in agreement with rezults from the intact locust muscle GABA receptors which have already been shown to selectively gate chloride ions.[3] Further experiments are needed to test the effects of antagonists and further clarify the role of chloride in the GABA response.

Application of 0.1 mM quisqualate to an oocyte clamped at -60mV also produced an inward current of up to 50 nA which occasionally had an oscillatory component. The reversal potential of this response was more variable and the mean value of some -10 mV would suggest that more than one ion may be involved. From initial ionic subsitution experiments (replacing 66mM of NaCl with choline chloride), Na^+ has been found to affect the size of the response. Oscillatory membrane currents in oocytes are indicative of the activation of Ca^{2+}-dependent Cl^- channels, thereby suggesting that the quisqualate response observed probably also involves a rise in intracellular Ca^{2+} activity. In contrast, membrane currents induced by glutamate were on the whole 'smooth' and had a reversal potential of around -61 mV. These results suggest that again more than one ion are involved, but these are at least quantitatively different to quisqualate-activated ionic current(s).

It is concluded that the Xenopus oocyte system would be highly suitable for multi-disciplinary study of insect neuromuscular transmitters.

REFERENCES

1 Gurdon, J.B., Lane, C.D., Woodward, H.R. and Marbaix, G. Nature. 1971, 253 177-182.

2 Barnard, E.A., Miledi, R. and Sumikawa, K. Proc.R.Soc.Lond.B. 1982, 215 241-246.

3 Usherwood, P.N.R. and Grundfest, H. J. Neurophysiol. 1965, 28 497-518.

4 Usherwood, P.N.R., In "Glutamate as a neurotransmitter." Editied by Dichiara. G., and Gessa. D.L. (1981). Raven Press. New York.

5 Usherwood, P.N.R. and Machili, P. J. exp. Biol. 1968, 40 341-361.

6 Barnard, E.A. and Bilbe, G. In "Neurochemistry, A Practical Approach" (1987). IRL Press:Oxford.

7 Barth, L.G. and Barth, L.J. J. Embryol. Exp. Morph. 1959, 71 210-222.

INHIBITION OF GLUTAMATE RECEPTOR BINDING BY DRUGS AND TOXICANTS.

Daniel B. Gant, Mohyee E. Eldefrawi and Amira T. Eldefrawi.

Dept. of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, Maryland, USA.

Rat brain glutamate receptors were identified by radioligand binding of [^{3}H]glutamate and the noncompetitive blockers [^{3}H]MK-801 and [^{3}H]PCP to synaptic membranes. Specific binding of [^{3}H]glutamate (50 mM Tris-acetate, pH 7.4, 0-4°C) was saturable with a single binding affinity, K_d = 288 nM, and a B_{max} of 5.84 pmol/mg protein. Displaceable binding of [^{3}H]glutamate to the filters, due to bacterial uptake, was eliminated by the addition of 0.05% Na azide and filtration of the buffer through sterilization filters. The allosteric blockers PCP, MK-801 and philanthotoxin had no effect on [^{3}H]glutamate binding, but the calcium channel blocker diltiazem reduced B_{max}. Inhibition of [^{3}H]glutamate binding was stereoselective among the agonists l- and d- glutamate > aspartate > homocysteate. Quisqualate inhibited binding with a K_i of 68 µM, while NMDA, domoic acid, APV, APB, and AMPA had K_i > 300 µM. [^{3}H]MK-801 binding was potentiated by glutamate and was saturable with a K_d of 7.68 nM and a B_{max} of 2.25 pmol/mg protein. The glutamate-potentiated binding of [^{3}H]MK-801 was inhibited in a mixed competitive-noncompetitive manner by philanthotoxin.

The crayfish abdominal muscle glutamate receptors were also identified by radioligand binding of [^{3}H]glutamate. There was no detectable specific binding of [^{3}H]MK-801. [^{3}H]Glutamate binding displayed a single binding site with a K_d and B_{max} of 103 nM and 3.93 pmol/mg protein, respectively. Inhibition by acidic amino acids was stereoselective among the l- and d- glutamate > aspartate > homocysteate. Quisqualate inhibited binding with a K_i of 106 μM, while NMDA, domoic acid, APV, APB, and AMPA had K_i > 300 μM. [^{3}H]PCP binding to crayfish abdominal muscle was species specific and was not modulated by glutamate or diltiazem. MK-801 inhibited [^{3}H]PCP binding with a K_i of 11.1 μM. The specific binding was saturable with a K_d of 18.8 nM and a B_{max} of 4.52 pmol/mg protein. (Supported by NIH Grant ES 02594)

DOPAMINE RELEASE BY COCKROACH SYNAPTOSOMES

*Andrew N. Gifford, Russell A. Nicholson and Paul E. Burt.

Wellcome Research Laboratories, Berkhampsted, Herts, England HP4 2DY
*Gatty Marine Laboratory, St Andrews, Fife, Scotland KY16 8LB.

Several lines of evidence indicate that dopamine (DA) may be a neurotransmitter in the CNS of insects. It has been shown to be present in the insect CNS by both fluorescent histochemical techniques and by radioenzymic assays[1] and dopamine elicits excitatory responses when applied to an identified insect central neurone.[2,3] Dopamine has also been shown to be accumulated by insect synaptosomes.[4] We show here that dopamine fulfils another of the criteria for a role as a neurotransmitter - that of release from synaptic terminals by depolarization.

Synaptosomes were prepared from the cerebral ganglia of 120 cockroaches (Periplaneta americana) according to the method described previously.[4] The synaptosomes were loaded with either (^{3}H)DA or (^{3}H) choline, distributed between ten filter units (Whatman GF/B) and superperfused with insect saline (50ml) followed by saline (20ml) containing the compound of interest. Fractions (3ml) were collected over a period of 30 minutes and their radioactivity determined using a scintillation counter.

Potassium (65mM) induces a small release of label in the synaptosomes incubated with (^{3}H)DA (fig. 1a) although this is considerably smaller than the release of label by the synaptosomes incubated with (^{3}H) choline. Measuring the amount of radioactivity released by lysing the synaptosomes with distilled water shows that this can be accounted for by a lower uptake of the (^{3}H)DA. The release of label by the synaptosomes incubated with (^{3}H)DA is greatly reduced by depolarizing them in the absence of calcium.

The calcium ionophore A23187, at a concentration of 10μM, (dissolved in 5μl DMSO) causes a large release of label from synaptosomes loaded with (^{3}H)DA (fig. 1b). The release is considerably reduced in the absence of calcium.

In mammalian systems, amphetamine is taken up into biogenic amine containing nerve terminals and displaces the biogenic amines from their storage sites, leading to their release into the extracellular medium. Amphetamine (100μM) induced a very large release of label from insect synaptosomes loaded with (^{3}H)DA (fig. 1c). Interestingly amphetamine does not lead to depletion of biogenic amines when injected into the whole insect,[5] perhaps because it fails to reach the nerve terminals.

Reserpine depletes biogenic amines in the CNS of both vertebrates and invertebrates.[6] In cockroaches this occurs over a time course of several hours. Reserpine, at a concentration of 15μM, (dissolved in 10μl DMSO) caused a small release of label from the insect synaptosomes, with a slower time course than with high potassium, A23187 and

amphetamine (Fig. 1d).

Control experiments, in which the compounds were replaced by saline containing 10ul DMSO, showed no release of label.

In conclusion these results demonstrate that insect synaptosomes loaded with (^{3}H)DA release label on depolarization and that this release is partially calcium dependent. This provides further evidence that dopamine is a neurotransmitter in insects and as in mammals can be induced by amphetamine and by reserpine.

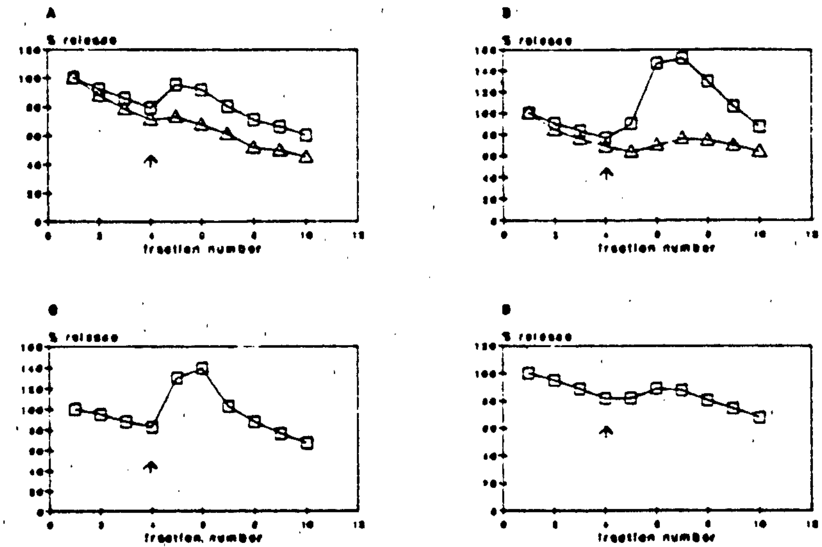


Figure 1. Release of label from synaptosomes loaded with (^{3}H)DA evoked by a) 65mM potassium, b) 10μM A23187, c) 100μM amphetamine, d) 15μM reserpine. Arrow indicates the point at which the compounds were added. Data are means of from 4 - 11 experiments. Squares are normal saline, triangles no calcium saline.

References

1. Evans, P.D. Adv. Insect Physiol. 1980 15, 317-473.

2. Pitman. R.M. Comprehensive Insect Physiology, Biochemistry and Pharmacology. Ed. Kerkut, G.A. and Gilbert, L.I. Pergamon Press, Oxford. 1985 11, 5-54.

3. Pitman, R.M. and Fleming, J.R. Neuropharmacology and Pesticide Action (Neurotox '85). London: Society of Chemical Industry. 147-150.

4. Gifford, A.N., Nicholson, R.A., Burt, P.E. and Pitman, R.N. Biochem. Soc. Trans. 1987 15, 885-886.

5. Omar, D., Murdock, L.L. and Hollingworth, R.M. Comp. Biochem. Physiol. 1982 73C, 423-429.

6. Sloley, D.B. and Owen, M.D. Insect Biochem. 1982 12, 469-476.

ROLE OF THE CATECHOLAMINERGIC AND SEROTONERGIC SYSTEM IN THE REGULATION OF THE DEVELOPMENT OF LOCUSTA MIGRATORIA

László Hiripi

Balaton Limnological Research Institute of the Hungarian Academy of Sciences, 8237 Tihany, Hungary

In the central nervous system of vertebrates the selective lesioning of the catecholaminergic and serotonergic pathways by the neurotoxin 6-hydroxydopamine /6OHDA/ and 5,7-dihydroxytryptamine /5,7-DHT/ respectively, is proved to be a useful tool for the functional studies of these aminergic system. We use these neurotoxins to study the role of the dopaminergic and serotonergic system in the development of Locusta migratoria.

Fifth larvae, staged with an accurancy of ± 4 hr, were treated on the 1st and 3rd days after moult. 6OHDA or 5,7DHT dissolved in 0.2 mg/ml ascorbic acid were injected in a volume of 20 ul into the abdomen. Control animals received the same volume of ascorbic acid. The dose of the 6OHDA or 5,7DHT were 500 ug/ /animal. The insect were fed on wheat seedlings and the animals weighed daily. In the cerebral ganglia the dopamine and serotonin as well as their metabolites the N-acetyl-dopamine and the N-acetyl-serotonin respectively, were measured by HPLC-ED method. The moulting of the animals were observed in each hour of the light period /12 h/.

The 5,7-dihydroxytryptamine decreased the concentration of both serotonin and N-acetyl-serotonin moderately in the ganglia. However, it had no toxic effect, it did not alter the body-weight gain, the feeding behaviour and the larvae lasted for 10 days as that of the control.

The 6OHDA caused a transient decrease the concentration of both dopamine and N-acetyl-dopamine in the cerebral ganglia. In the control animals the body-weight doubled by 8 days after the moult while in the 6OHDA treated animals the body-weight

doubled by 17 days after moult. 50% of the larvae died and the survive larvae lasted 18 days insted of 10 days. During that period when the body weight of the animal is unchanged the food consumption decreased significantly.
The same effect was observed when the adult animal was treated with 6OHDA.
The results suggest that neither the serotonergic nor dopaminergic system are involved in the hormonal control of moulting. However the dopaminergic system is involved in the regulation of feeding behaviour. The chemical lesioning of the dopaminergic system caused the growth disruption by the feeding inhibition which leaded to high mortality.

THE METABOLISM OF NEUROPEPTIDES BY MEMBRANE PEPTIDASES FROM THE LOCUST, *SCHISTOCERCA GREGARIA*

R.Elwyn Isaac
Department of Pure and Applied Biology
University of Leeds
Leeds LS2 9JT

In vertebrate tissues, extracellular membrane enzymes are considered to be involved in the inactivation of neuropeptide signals and it appears likely that a few peptidases, positioned on the plasma membrane, are responsible for the hydrolysis of a range of peptide messengers.[1]

In recent work from this laboratory two insect neuropeptides, proctolin and locust adipokinetic hormone (AKH), have been used as substrates to characterise membrane enzymes from the CNS of the locust, *Schistocerca gregaria*. It would appear that neuropeptides can have diverse functions and it has been suggested that proctolin and AKH-like peptides may play a role in neurotransmission in the central nervous system of insects, in addition to their effects on peripheral tissues. The metabolism of proctolin (Arg-Tyr-Leu-Pro-Thr) and AKH (pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2) by neural membranes was studied using reverse phase hplc to isolate and characterise degradation products.

Nervous tissues from the locust are rich in aminopeptidase activity that degrades proctolin to yield Tyr-Leu-Pro-Thr.[2] Much of this activity is associated with a washed neural membrane preparation (a 30K g pellet) and appears to be responsible for the primary hydrolysis of proctolin when incubated *in vitro* at a substrate concentration of 100μM. This aminopeptidase activity had a pH optimum of 7.0, an apparent Km of 23μM and was strongly inhibited by both amastatin (IC_{50} 0.3μM) and by EDTA (1mM).

At proctolin concentrations below 1μM, the dipeptide Arg-Tyr was also detected as a significant metabolite and appeared to result from the cleavage of the Tyr-Leu bond. This weak activity was also maximal at pH 7.0 and displayed high affinity for proctolin with an apparent Km of around 0.35μM.

By separating the crude neural membranes into synaptosomal and mitochondrial fractions, it was shown that the aminopeptidase activity, like acetylcholinesterase, was enriched in synaptosomal membranes. The Tyr-Leu hydrolysing activity on the other hand appeared to be predominantly (66%) associated with the mitochondrial membrane fraction.[3]

AKH is metabolised by neural membranes releasing two fragments (A&B). The addition of amastatin to the incubation mixture inhibited the formation of A whilst the amount of B produced was increased proportionally, demonstrating that B was the primary hydrolysis product. Amino acid analysis identified B as (Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH2) indicating that removal of the N-terminal tripeptide by an endopeptidase is the first step in the degradation of AKH by neural membranes.

The activity did not appear to be enriched in a synaptic membrane preparation. However, recent experiments have shown that specific inhibitors can be used to differentiate between the activities associated with synaptic and mitochondrial membranes. Studies are now in progress to characterise the synaptic membrane enzyme.

The use of AKH as a substrate has demonstrated that synaptic membrane preparations are able to degrade a peptide that is protected from exopeptidase attack by the presence of blocked terminal amino acids. The bond specificty of the enzyme is not known but it is of interest to note that many of the neuropeptides recently isolated from insects possess, as in AKH, phenylalanine at position 4. This hydrophobic amino acid donates its amino group to the scissile bond of AKH.

The localisation of an aminopeptidase and endopeptidase activity in synaptic-membrane preparations is consistent with roles for these enzymes in the inactivation of synaptically released peptides. Similar peptidases have also been detected in membrane preparations from locust gut, fat body and Malpighian tubules suggesting that the same or similar enzymes to those described in the present report may have a general role in the inactivation of insect peptides.

1. Turner,A.J.;Matsas,R.; Kenny,A.J. Biochem.Pharmacol. 1985,34,1347-1356.
2. Isaac,R.E. Biochem.Soc.Trans.1985,13,1238-1239
3. Isaac,R.E. Biochem.J. 1987,245,365-370.

INTERCHANGE γ-AMINOBUTYRIC ACID (GABA) AND ELECTRICAL ACTIVITY OF THE BRAIN AFTER INJECTION THE PESTICIDES (MUGAN -I AND OTHERS).

KADYROV G.K.

Laboratory of Neurochemistry, Institute of Physiology Azerbaijan Academy of Sciences. Baku, USSR, 370602.

The experiments were conducted on rabbits species of chinchilla (the weights- 3-3,5 kg.).

The electrodes were implanted to the rabbits of their mesencephalon of reticular formations, sensemotor of cortex and cerebellum (on the coordinates of J.W.Everett and J.D.Green atlas).

Were determined both the electrical activity the named structure of the brain and investigated points of the interchanging GABA in the same structers (for the determing these points in the mesentsefalitical reticular formations of rats were taken the whole bole of the brain, beginnig from the medulla till the hypothalamus inclusivly).

The pesticides were applied one and many times in the wateralchal solution (intermuscular in the doze 2;4 and 6 mgr/kg /for rabbits/ and 0,2;0,4 and 0,6 mgr/100 gr mass of the animals /for rats/).

In the character of control was served intermuscular injection in those volume of the ethyl alcohol distilled water in which were dissolved the pesticides.

Intermuscular injection of this solution did not provoke the displacement.

Expression of decreasing the content of GABA, GA and AA were registered after 30 minutes injection pesticides accordingly in dozes of 6 mgr/kg and 0,6 mgr/100 gr mass of animals.

On the received experimental iata the pesticides after the penetration to the organism of animal change the activity of enzymes GAD (EC 4.1.1.15) and GABA-T (EC 2.6.1.19) in the investigated structures of the brain, which is stipulated accordingly the displacements in the content GABA, GA and AA. But the changing is not simple even for the investigated sections of the brain. These neurochemistry displacements were correlated with the studying the electrographical points of the same sections of the brain.

In particular electroencephalographical activity and provoked with this potentials in focus of maximum electrical activity in projection zone contralaterial sensmotor cortex in response to electronkins irritation of the rabbits.

In the light of the received data it offers define interes the researching the functional condition adreno-and cholinoergical structure of the brain after many days injections to the organism pesticides. The experiments showed, that in the pesticidized animals such a spesifical preparations as ephedrin and aminazin, galantamine and amizil were accordingly exiting and blockading adreno-and-cholinoergical structures in accord analysis the studying electrographical phenomenons did not be able gave their evidence (as it usually take place for the intacting animals for those, which did not receive pesticides). This fact, in my opinion, is very interesting, thou for the present it is diffilculty explicable or, in general, do not defying to the interpretation. The experiments in this direction are continueing.

Sawyer,C.M., Ewerett,J.W.,Green,J.D.comp.Neurol,1954,101, pp. 301-324.

MODULATION OF ACETYLCHOLINE RELEASE AND HIGH AFFINITY CHOLINE TRANSPORT IN INSECT SYNAPTOSOMES

M. KNIPPER, J. KRIEGER, H. BREER
University Stuttgart-Hohenheim
Institute of Zoophysiology
7000 STUTTGART 70, West Germany

Neurochemical aspects of cholinergic synapses are of particular interest for insect neuropharmacology. The release of acetylcholine (ACh) from isolated nerve terminals has been studied using a microperfusion system; it was found that the evoked Ca^{2+}-dependent release of neurotransmitters could be stimulated under in vitro conditions. Thus, synaptosomal preparations may be suitable to study the regulation of presynaptic activities. In the presence of cholinergic agonists, like carbamylcholine, a significant reduced rate of ACh-release was observed, suggesting an autoregulation of the release process. In series of perfusion experiments it was revealed, that the observed modulation of ACh-release was mediated by muscarinic autoreceptors and extended studies on the subtype of receptor involved in this regulatory process suggest that the modulatory effect of ACh is obviously transduced via M_2-type receptors. This receptor subtype is apparently located in the presynaptic membrane of cholinergic nerve terminals and negatively coupled to the adenylate cyclase; thus reducing the synaptosomal cAMP-level. Activation of appropriate heteroreceptors in isolated nerve endings, e.g. via octopamine, resulted in an activation of the synaptosomal adenylate cyclase and an increase of the synaptosomal cAMP-concentration: consequently an enhanced release rate for ACh was observed. These results suggest a regulatory role of intrasynaptosomal cAMP on the rate of transmitter release, probably via cAMP-dependent kinase reactions.

Phosphorylation of presynaptic membrane proteins may be considered as a critical step in modifying essential functional elements of the release machinery for neurotransmitters and thus modulating synaptic signal transmission. This view is supported by experiments using phorbolesters, which mimic the stimulatory effects of endogenous diacylglycerol on protein kinase C; activation of protein kinase C in isolated nerve terminals caused a significant enhancement on the evoked release of ACh, this effect was blocked by polymyxin, an inhibitor of protein kinase C.

The high affinity transport system for choline, which is supposed to be the rate limiting, regulatory element for the synthesis of ACh, was found to be

modulated in a simiIiar way via presynaptic auto- and heteroreceptors. In addition it was observed, that extracellular ATP specifically inhibits this transport system; the results of pharmacological experiments suggest that the regulatory effect of ATP is mediated via a kinase reaction, obviously via ektokinases located at cholinergic nerve terminals. This specific transport system for choline is obviously a symmetric carrier-system, which is energized by ion gradients, notably the sodium gradient. In attempts towards a more detailed characterization of the carrier-system, responsible for the high affinity accumulation of choline, tritiated hemicholinium-3 has been employed in binding studies on membrane preparations. As a first step towards a molecular identification of the carrier-polypeptides, monoclonal antibodies has been raised, which specifically blocked the high-affinity accumulation of choline and recognized a specific polypeptid in western blots; purification and reconstition experiments will reveal wether the identifed polypeptides can be considered as functional elements of the high affinity transport system for choline.

DETERMINATION OF THE MOLECULAR WEIGHT OF AN INSECT NA+ CHANNEL COMPONENT BY USING SCORPION TOXINS

Maria Elena de LIMA, François COURAUD, Bruno LAPIED*, Carlos Ribeiro DINIZ+, Hervé ROCHAT.
CNRS UA 1179, INSERM U. 172, Laboratoire de Biochimie, Faculté de Médecine Nord, Bd, Pierre-Dramard, 13326 Marseille Cédex 15, France.
* Laboratoire de Physiologie, Faculté de Médecine, rue Haute-de-Reculée, 49045 Angers, France.
+ Departamento de Bioquimica e Imunologia, I.C.B., Universidade Federal de Minas Gerais, 30000 Belo Horizonte, M.G., Brazil.

Using photoaffinity labeling with arylazide derivatives of four radioiodinated scorpion toxins active on Na^+ channel of insect it is shown that all the four toxins bind to the same molecular weight component of Mr 138,00 ± 12,000 (n = 17) of the nerve cord synaptosomal fraction of the cockroach (Periplaneta americana). Since the only high molecular weight in the vertebrate Na^+ channel is the Mr = 260,000 α-subunit (Catterall, W.A., 1986) and since an homologous protein has been shown to be present in Drosophila (Salkoff, L. et al., 1987) we may assume that the component labeled in our experiments is homologous to the α-subunit described in vertebrate tissues. One of the four toxins (Ts VII) used is also highly active in mammal. Ts VII is shown to label a low molecular weight component (31,000 ± 4,000) in rat brain synaptosomes, contrary to what we obtain with the insect preparation. However, this is in agreement with previous data showing that β-toxins specifically label β_1-subunit of the Na^+ channel of vertebrates (Jover, E. et al., 1987, in press).

References :

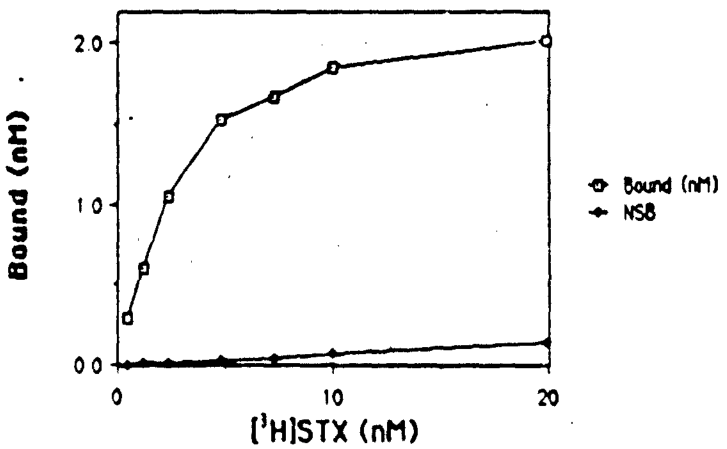
- Catterall, W.A. (1986) Ann. Rev. Biochem., 55, 953-985.
- Salkoff, L., Butler, A., Wei, A., Scavarda, N., Giffen, K., Ifune, C., Goodman, R., Mandel, G. (1987). Science, 237, 744-749.

PHARMACOLOGICAL PROPERTIES AND MOLECULAR SIZE OF INSECT SODIUM CHANNELS

Sarah C R Lummis and David B Sattelle
AFRC Unit of Insect Neurophysiology and Pharmacology, Department of Zoology, University of Cambridge, CB2 3EJ, UK.

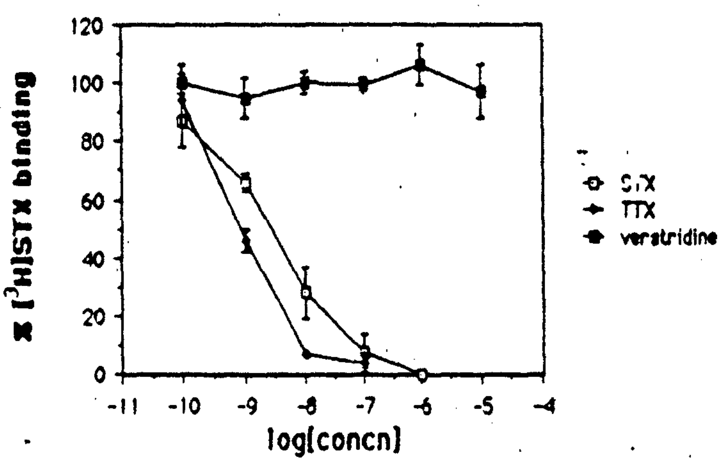
Voltage-dependent sodium channels are selectively blocked by the marine neurotoxin saxitoxin (STX), which has proved extremely useful in the isolation and characterization of these transmembrane proteins[1]. Electrophysiological studies have demonstrated that this toxin is a potent blocker of cockroach (*Periplaneta americana*) sodium channels, effective at nanomolar concentrations[2]. Membrane extracts from the central nervous system of the same species of cockroach showed a saturable component of specific [^{3}H]STX binding. Non-specific binding was determined using 1μM unlabelled STX and represented less than 10% total binding. Analyses of the binding data showed a K_d (dissociation constant) of 2.25 ± 0.52 nM and B_{max} (maximum number of binding sites) of 12.9 ± 1.4 pmol/mg protein.

Figure 1: Specific binding of [^{3}H]STX to a membrane extract from the CNS of the cockroach (*Periplaneta americana*).



The pharmacological characteristics of [^{3}H]STX binding revealed that the specific binding component was blocked by STX and TTX, but not by veratridine or deltamethrin, which act at different sites on the channel molecule. The effectiveness of unlabelled STX samples prepared from different sources (*Mytilus*, *Saxidomus* and *Gonyaulax*) were compared as inhibitors of [^{3}H]STX binding. By means of radiation inactivation and simple target theory, it is possible to determine the molecular target size of membrane proteins. In this way the molecular target size of the cockroach [^{3}H]STX binding site was estimated under a variety of conditions to yield information about the molecular organisation of the insect sodium channel protein.

Figure 2: Inhibition of [^{3}H]STX binding to cockroach nerve cord membranes by unlabelled STX, TTX and veratridine.



References

1. Yamamoto, D. Prog. Neurobiol. 1985 24 257-291

2. Sattelle, D.B., Pelhate, M. and Hue, B. J. exp. Biol. 1979 83 41-48

AN ANTISERUM TO A PEPTIDE SEQUENCE DERIVED FROM A CLONED LOCUST cDNA CROSS REACTS WITH THE NATIVE LOCUST NICOTINIC ACETYLCHOLINE RECEPTOR

David R.E MacAllan*, John Marshall+, George G.Lunt* and Eric A.Barnard+

*Department of Biochemistry, University of Bath, Bath England, BA2 7AY

+MRC Molecular Neurobiology Unit, Addenbrookes Hospital, Cambridge England

It is becoming increasingly clear that neuronal nicotinic acetylcholine receptors (nAChR's) in mammalian brain are coded by genes[1], that are related to those coding for the well characterised peripheral receptor but the receptor proteins exhibit different pharmacological specificities[2]. It is also suggested that the nAChR may be a member of an even larger superfamily of ion channel receptors[3,4]. A close examination of the amino acid sequence reveals that there is homology between the different subunits of the nAChR and also between any one of the nAChR subunits and the $GABA_A$ and glycine receptor subunits. The most highly conserved region in these receptors appears to be a sequence which is able to form a disulphide loop in the N terminal region of the extracellular domain. Such a sequence has been identified in a cDNA clone from Drosophila melanogaster, which is thought to code for a putative insect neuronal nAChR subunit[5]. We have identified an identical sequence in a clone isolated from a genomic library of the locust Schistocerca gregaria[6], and have synthesised a peptide containing this disulphide loop sequence. The peptide was synthesised with cysteine residues covalently modified with an acetamidomethyl group protecting the side chains. An antiserum was raised against the peptide which recognised a protein of M_r 49,000 in a Western blot using a locust ganglion membrane fraction[7] as the antigen. The antiserum also identified a single major band in a Western blot with

the αbungarotoxin binding component purified from the same membrane preparation. Furthermore, an antiserum raised against the purified αbungarotoxin binding component recognised the synthetic peptide in a dot blot immunobinding assay. We have previously reported the purification of the αbungarotoxin binding component from this tissue which had a major subunit of M_r 49,000 and have shown that a protein with this molecular weight in the membrane fraction is labelled by the nicotinic affinity ligand 4-(N-maleimide)-[^{3}H]benzyltrimethylammonium[8].

We conclude that the protein of M_r 49,000 which is recognised by the antiserum to the synthetic peptide is a subunit of the insect nAChR.

1. Goldman, D., Deneris, E., Luyten, W., Kochhar, A., Patric, J. and Heinemann, S. Cell 1987 48 965-973

2. Morley, B., Dwyer, D., Strang-Brown, P., Bradley, R. and Kemp, G. Brain Research 1981 262 109-116

3. Grenningloh, G., Rienitz, A., Schmitt, B., Methfessel, C., Zensen, M., Beyruther, K., Gundelfinger, E. and Betz, H. Nature 1987 328 215-220

4. Schofield, P., Darlison, M., Fujita, N., Burt, D., Stephenson, F., Rodriguez, H., Rhee, L., Ramachandran, J., Reale, V., Glencorse, T., Seeburg, P., and Barnard E. Nature 1987 328 221-227

5. Hermans-Borgmeyer, I., Zopf, D., Ryseck, R-P., Hovemann, B., Betz, H. and Gundelfinger, E. EMBO J. 1986 5 1503-1508

6. Marshall, J., Darlison, M., Lunt, G. and Barnard, E. Biochem Soc Trans. 1988 (in press)

7. Filbin, M., Lunt, G. and Donnellan, J. Eur. J. Biochem. 1983 132 151-156

8. MacAllan, D. and Lunt, G. Biochem. Soc. Trans. 1986 14 610-611

CLONING AND EXPRESSION OF THE NICOTINIC ACETYLCHOLINE RECEPTOR FROM THE LOCUST SCHISTOCERCA GREGARIA

J. Marshall, D.B. Sattelle*, J.A. David*, M.G. Darlison, G.G. Lunt+ and E.A. Barnard

MRC Molecular Neurobiology Unit, MRC Centre, Hills Road, Cambridge CB2 2QH

*AFRC Unit of Insect Neurophysiology and Pharmacology, Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ

+Department of Biochemistry, University of Bath, Bath BS2 7AY

In vertebrates it has been demonstrated that the neuronal nicotinic acetylcholine receptor (nAChR) is composed of two different polypeptide chains, the alpha and beta subunits (1). In invertebrates some biochemical studies have suggested that the nAChR in the central nervous system (CNS) is a homo-oligomeric structure (2). It has been proposed therefore that it is an evolutionary ancestor of the vertebrate nAChR. Molecular cloning techniques, however, have provided preliminary evidence for multiple nAChR genes in the locust Schistocerca gregaria (3). In order to investigate further the oligomeric structure of the locust Schistocerca gregaria nAChR we have isolated cDNA clones encoding nAChR polypeptides and used them in conjunction with the the Xenopus oocyte expression system.

Poly(A)$^+$ RNA was purified from locust embryos. This mRNA was to construct a λgt10 cDNA library. This library was screened under low stringency using a 400-basepair fragment of a previously cloned locust nAChR gene (3). This screen yielded two non-identical cDNA clones (ARL 1 and ARL 2) which show sequence homology to a known cDNA of the Drosophila neuronal nAChR (4). We tentatively identified one of these clones as an α and the other as a non-α nAChR polypeptide.

ARL 1 and ARL 2 have been used in expression studies of the respective RNAs by means of micro-injection into *Xenopus* oocytes. Clearly defined electrophysiological responses to bath-applied nicotine were found in injected oocytes, with a threshold at 1.0×10^{-8} M. No nicotine sensitivity was observed in control non-injected oocytes.

In summary, we have identified different cDNA clones from the locust *Schistocerca gregaria*. Expression studies indicate that these genes encode a functional nicotinic acetylcholine receptor.

J.M. acknowledges the support of Shell Research Ltd., U.K.

References

1. Boulter, J., Connolly, J., Deneris, E., Goldman, D., Heineman, S. and Patrick, J. (1987) PNAS 84, 7763-7767
2. Hanke, W. and Breer, H. (1986) Nature 321, 171-174.
3. Marshall, J., Darlison, M., Lunt, G. and Barnard, E.A. (1988) Biochem. Soc. Trans. (in press).
4. Hermans-Borgmeyer, I., Zopf, D., Ryseck, R-P, Hovemann, B., Betz, H. and Gundelfinger, E.D. (1986) EMBO J. 5, 1503-1508

INTERACTION OF GOSSYPOL WITH GLUTAMATE, ACETYLCHOLINE AND ADRENERGIC RECEPTORS.

Nabil A. Mansour, Shebl M. Sherby, Ahmed M. El-Nabaway, and Mamdouh H. Idriss.

Department of Plant Protection, Division of Pesticide Chemistry, Faculty of Agriculture, University of Alexandria, Alexandria, Egypt.

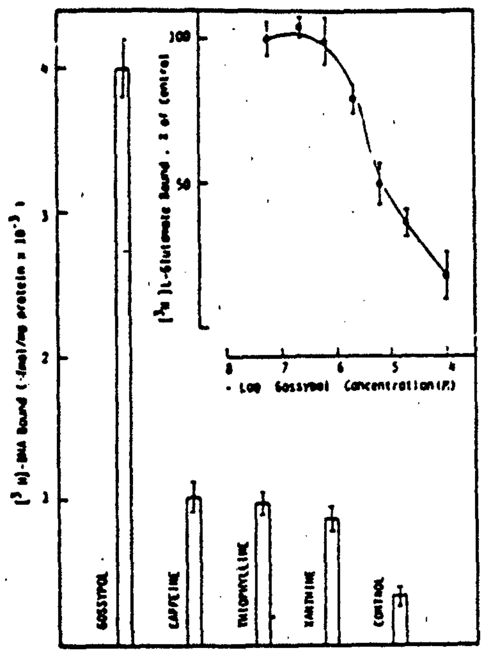
We have recently found that gossypol causes infertility and hormonal imbalance on some lepidopterous insects[1,2,3]. We have investigated its interaction with certain receptor systems. Binding of [^{3}H]glutamate to rat brain synaptic membrane was carried out using the filtration assay[4]. Using Tris-citrate buffer, pH 7.4 in presence of 2.5 mM Ca^{2+}, a single affinity site was detected for [^{3}H]glutamate binding to rat brain synaptosomes, with K_D of 0.33 ± 0.05 μM and B_{max} of 57.0 ±10.0 pmol/mg protein. Binding was inhibited by gossypol with k_i value of 5.8 ± 0.4 μM. This binding was dramatically increased in absence of Ca^{+2}. Although, gossypol at low range of concentrations stimulated [^{3}H]glutamate binding, at higher concentrations (<30 μM), the stimulation was reduced.

The effects of gossypol on the binding of ligands to the nicotinic acetylcholine receptor (n-AChR) of *Torpedo* membranes were studied. Gossypol, at concentrations ranging from 0.1 to 100 μM, increased the binding of [^{3}H] H_{12}-HTX and [^{3}H]PCP at 2 nM to *Torpedo* membranes, similar to carbamylcholine, in a dose-dependent manner, up to 80 - and 40 fold for both ligands, respectively.

Binding of -[^{3}H]dihydroalprenolol, (-[^{3}H]DHA), a potent β-adrenergic antagonist, was used to identify β-adrenergic receptors of membrane preparation from frog erythrocytes and *Philosamia ricini* prothoracic glands by filter assay technique[5]. Gossypol was more potent than xanthine derivatives in stimulation (-)[^{3}H]DHA binding to its receptor.

It is concluded that gossypol has two concentration-dependent effects on the glutamate, n-AChR, and β-adrenergic receptors. At low concentrations, gossypol like agonists potentiate binding of the correaponding specific ligands to glutamate and β-adrenergic receptors, while such potentiation occurred only to the channel

Stimulation of (-) [^{3}H] DHA binding to frog erythrocytes β-adrenergic receptors by gossypol & xanthine derivatives.
In set:
Inhibition of [^{3}H] L-Glutamate specific binding to synaptosomal preparation of rat brain by gossypol.



drugs of n-AChR. It is suggested that gossypol induces a receptor conformation that modulate the agonist affinity. It do so by binding to the lipid bilayer, or to certain sites that may be at the domains of certain protein in the lipid bilayer. Inhancement of occupying the receptor binding site(s) in the presence of gossypol might have an effect on another component after forming a transient complex with the system. The effect on the adenylate cyclase-coupled β-adrenergic receptors might explain that gossypol interfere with the cAMP g ormation system which antagonizes the ecdysone action on its target, which would affect the insect fertility.

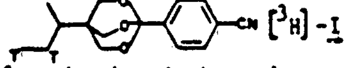
1. Mansour, N.A., Sherby, S.M., Morshedy, M.F., and Idriss, M.H. The 5th Intern. Cong. Pestic. Chem.,1982, Kyoto, Japan.
2. Sherby, S.M., Morshedy, M.F., Idriss, M.H., and Mansour, N.A. J. Coll. Agric. King Saud Univ. 1987 9 317-329
3. El-Sebae, A.H., Sherby, S.M., and Mansour, N.A. J. Environ. Sci. Health--, PESTIC., FOOD CONTAM, AGRIC. WASTES. 1980 B11 1-11
4. Sherby, S.M., Eldefrawi, M.E., Wafford, K.A., Sattelle, D.B., and Eldefrawi, A.T. Comp. Biochem. Physiol. 1987 87C 99-106
5. Lefkowitz, R.J., Caron, M.G., Michel, T., and Stadel, J.M. Federation Proceedings 41 2664-2670

4-s-[^{3}H]BUTYL-1-(4-CYANOPHENYL)-2,6,7-TRIOXABICYCLO[2.2.2]OCTANE: SPECIFIC BINDING IN THE AMERICAN COCKROACH CENTRAL NERVOUS SYSTEM.

Russell A. Nicholson, Christopher J. Palmer* and John E. Casida*.

Wellcome Research Laboratories, Berkhamsted, Hertfordshire, U.K. and *Pesticide Chemistry and Toxicology Laboratory, Department of Entomological Sciences, University of California, Berkeley, California 97420, U.S.A.

The title compound (I) was prepared as a potential radioligand probe for a site of insecticidal action in the insect central nervous system (CNS). The 4-t-butyl analogue of I is a much more potent insecticide[1] than 4-t-butyl-1-phenyl-2,6,7-trioxabicyclo[2.2.2]octane, bicyclophosphorus esters and picrotoxinin which were used earlier as radioligands or prototypes thereof. [^{3}H]-I was prepared with a specific activity of 60 Ci/mmol.


[^{3}H]-I

The binding characteristics of [^{3}H]-I were investigated using a membrane fraction isolated from head and thoracic ganglia of the cockroach (Periplaneta americana). Nervous tissue from 80 male cockroaches was homogenised in 25ml. Van Harreveld's saline[2] (15 excursions; motor driven pestle) and centrifuged (480g; 10 minutes). Centrifugation of the resulting supernatant (35,000g; 35 minutes) yielded a pellet which was resuspended in a further 30ml. saline and centrifuged (35,000g; 35 minutes). Washed membranes were resuspended in saline (sodium chloride 200mM; sodium phosphate 5mM, buffered to pH 7.7) to give a protein concentration of 1mg/ml. Routine binding assays were initiated by introducing fresh membrane suspension (100µl) into saline (900µl) containing radioligand (1.3nM) in the presence or absence of displacer. Incubations were carried out at 22°C for 30 minutes whereupon ice-cold saline (2ml.) was added and membrane-bound radioactivity was immediately separated from the medium by vacuum filtration on Whatman GF/C filters. Filters were quickly washed with ice-cold saline (2x5ml.) and radioactivity quantitated using liquid scintillation counting. Specific binding was defined as the difference between total binding and binding in the presence of 6µM unlabelled I. Specific binding (which represents 84.4%±3.7 of total binding) is essentially saturated at a radioligand concentration of 2.8nM. This represents a maximum number of binding sites of 0.1 pmol/mg protein. Under standard assay conditions the IC_{50} for displacement of [^{3}H]-I by unlabelled I is between 10 and 20nM (see Fig. 1). The $t_{½}$ for association at 22°C is approximately one minute and full equilibration is achieved by 30 minutes, although at 0°C the association rate is considerably reduced ($t_{½}$=30 minutes). After preequilibration the dissociation of [^{3}H]-I by unlabelled I followed a biphasic course at 22°C. Substitution of chloride ions by acetate clearly demonstrated a minimal dependence of binding on chloride ion concentration.

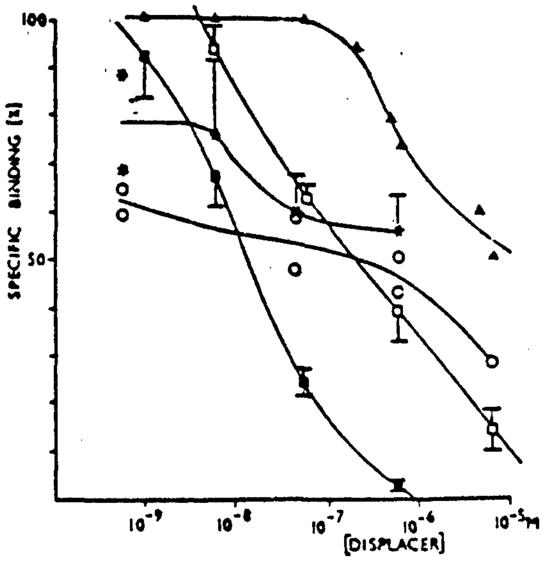


Figure 1. Effect of unlabelled I (■), 4-t-butyl-3-cyano-1-(4-ethynylphenyl)-2,6,7,trioxabicyclo[2.2.2]octane (□), picrotoxinin (▲), lindane (✱) and ivermectin (O) on specific binding of [^{3}H]-I to cockroach CNS. Dieldrin gave 16±2% and 30±3% displacement of [^{3}H]-I at 60nM and 0.6µM respectively.

4-t-Butyl-3-cyano-1-(4-ethynylphenyl)-2,6,7-trioxabicyclo [2.2.2]octane (a highly potent inhibitor of the GABA-gated chloride channel in mammalian brain preparations[3]) displaced [^{3}H]-I in a dose dependent manner(IC_{50} 0.2μM). Less potent displacement effects were observed with picrotoxinin and dieldrin (Fig.1) whilst GABA produced a weak inhibition of binding at concentrations of 0.1mM and above. Although unusually shallow inhibition curves were obtained with lindane and ivermectin both neurotoxicants were able to produce significant inhibition of binding at sub-nanomolar concentrations. The α-cyano-pyrethroid deltamethrin and bicyclophosphorothionate TBPS failed to displace specifically bound I at 0.6μM.

These results demonstrate that at nanomolar concentrations the novel radioligand [^{3}H]-I binds to receptors in membrane preparations isolated from insect CNS in a reversible, temperature-sensitive and chloride-independant manner. The recognition site for I appears to be associated with the chloride-ion selective channel as several classes of neurotoxicants reported to interact with this complex[3-7] were shown to displace binding Separate investigations reported at this meeting using insect synaptosomes and dissociated neuronal somata provide further evidence that the central actions of bicycloortho-carboxylates in insects are mediated at the chloride channel complex.

Acknowledgement: supported in part by US NIH Grant PO1 ES00049

1. Palmer, C.J., Casida, J.E. J. Agric. Food Chem. 1985 33 976-980

2. Van Harreveld, A. Proc. Soc. Exp. Biol. Med. 1936 34 428-432

3. Palmer, C.J., Casida, J.E. Unpublished results

4. Takeuchi, A., Takeuchi, N. J. Physiol. 1969 205 377-391

5. Matsumoto, K., Yamazaki, J., Kasuya, M., Fukuda, H. Neuroscience Letts. 1986 69 279-284

6. Tanaka, K., Matsumura, F. Pestic. Biochem. and Physiol. 1985 24 124-135

7. Ghiasuddin, S.M., Matsumura, F. Comp. Biochem. Physiol. 1984 73 141-144

PRESYNAPTIC ACTIONS OF KAINIC ACID AT THE ISOLATED MAMMALIAN GLUTAMATERGIC NEURONE

Jennifer M. Pocock*, Helen M. Murphie and David G. Nicholls

Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland, U.K.

Kainic acid, one of the most potent of the amino acid neuroexcitants, is a conformationally restricted analogue of glutamate, acting on specific receptors in the mammalian central nervous system to cause a long-lasting neuronal depolarization[1]. Much focus has been centred on the possible presynaptic actions of this neurotoxin[2].

We have recently developed a continuous fluorometric assay for glutamate release from synaptosomes[3,4] which enables a clear distinction to be made between Ca^{2+}-independent glutamate release from the cytoplasm and Ca^{2+}-dependent exocytotic release from non-cytoplasmic, probably vesicular pools. Use of this assay has allowed us to resolve some of the complexities surrounding the presynaptic actions of kainic acid[5].

We show that kainate inhibits the exchange of D-aspartate into guinea-pig cerebrocortical synaptosomes, inhibits the Ca^{2+}-independent efflux of endogenous glutamate in the presence of a trapping system for the released amino acid, but potentiates a Ca^{2+}-independent net efflux of endogenous

*present address: Department of Entomology, University of California, Riverside CA 92521-0314 U.S.A.

and labelled glutamate and aspartate in the absence of the trap. Dihydrokainate has a similar effect. No discrepancy is seen between the release of endogenous and exogenously accumulated amino acid.

We conclude that the presynaptic effects of millimolar kainate on guinea-pig cerebrocortical synaptosomes can be explained simply by the inhibition of the plasma membrane Na^+:glutamate (aspartate) cotransporter. This effect is Ca^{2+}-independent and does not influence the exocytotic, transmitter pool of glutamate, release of which is not affected by the neurotoxin.

1. Cooper, J. R., Bloom, F. E. and Roth, R. H. (1986) The Biochemical Basis of Neuropharmacology, Oxford University Press, New York/Oxford
2. Coyle, J. T. (1983) J. Neurochem. 4 1-11
3. Nicholls, D. G. and Sihra, T. S. (1986) Nature 321 772-773
4. Nicholls, D. G., Sihra, T. S. and Sanchez-Prieto, J. (1987) J. Neurochem. 49 50-57
5. Pocock J. M., Murphie H. M. and Nicholls D. G. (1988) J. Neurochem. (in press)

QUANTITATIVE THREE-DIMENSIONAL MORPHOLOGICAL ANALYSIS OF LOCUST CNS GABA RECEPTORS

Leo P. Schouest, Jr.[+], Thomas A. Miller[+] and Richard W. Olsen[•]

[+]Department of Entomology, University of California, Riverside, California 92521
[•]Department of Pharmacology, University of California, Los Angeles, California 90024

We used receptor autoradiography for the specific localization and quantitative characterization of insect CNS GABA receptors using several GABA agonists and antagonists as competitive binders to ^{3}H-muscimol. The compounds used in this specificity experiment were: muscimol (1 uM), gamma-aminobutyric acid (50 uM), isoguvacine (1 uM), nipecotic acid (50 uM), avermectin $B1_a$, and trans aldrin (50 uM). Competitive binding results from these compounds were compared to CNS sections incubated in 40 nM ^{3}H-muscimol (Amersham) alone at the same time. Since adjacent cryostat sections of locust brain and thoracic ganglia were utilized in the incubation procedure[1], differently treated sections were roughly anatomically equivalent since 35um encompassed each series. Briefly, locust brain or thoracic ganglia (*Schistocerca americana*) was dissected out in a cold Na^+-free buffer, briefly rinsed in fresh cold buffer and quickly frozen in OCT compound for sectioning. Sections were cut at 5um and placed on gelatin subbed slides and stored at -20°C until needed. Slides were incubated in each solutions for 40 minutes. Once dried the slides were exposed to LKB Ultrafilm for 12 weeks at 4°C. Film was conventionally developed and then analyzed. Analysis of the autoradiogram images consisted of first enlarging the small image and then digitizing the image by scanning the autoradiograph via a linear photodiode array 12-bit CCD camera using a frame transfer method (Microscan 1000). Digitized images were stored and transferred to an image analysis program (Imagemaster 1000) where the quantitated image was edited and analyzed. A four-quadrant image board allowed comparisons between control and adjacent treated sections by storing four images at once. Tritium standards (Amersham) placed with the slides during the film exposure period provided quantitative calibration of optical density values. Composite images consisting of control, treated and standards were constructed, stored as a new image thus allowing direct readings of optical density from each autoradiographic image. Optical density readings were automatically converted to DPM based on a standard curve obtained from digitizing calibration standards. Three-dimensional images of brain and thoracic ganglia were compiled from concurrent adjacent sections and transferred into a three-dimensional reconstruction program. Our results indicate competitive binding of GABA receptors was seen for muscimol, isoguvacine, avermectin and aldrin. Overlay subtraction of images showed specific GABA receptor locations for the lamina ganglionaris, some portions of the medulla and lobula plate as well as the olfactory glomerulus. GABA receptors were also seen to occur in all three thoracic ganglia in small specific regions. Three-dimensional reconstruction allows the quick visualization of neuronal regions specific for various receptor types.

1. Lunt, G.G., Robinson, T., Miller, T., Knowles, W P. and Olsen, R.W. Neurochem. Int. 1985 7 751-754.

AN L-GLUTAMATE BINDING SITE IN THE CENTRAL NERVOUS SYSTEM OF PERIPLANETA AMERICANA.

Maria-Isabel Sepulveda and David B. Sattelle.

A.F.R.C. Unit of Insect Neurophysiology and Pharmacology. Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, U.K.

There is considerable evidence that L-glutamate is an excitatory neurotransmitter at arthropod neuromuscular junctions[1]. Effects of glutamate have been described[2] for an identified neurone in the central nervous system (CNS) of the cockroach (*Periplaneta americana*)

The data currently available for insects are not all easily reconciled with the currently accepted classification of vertebrate glutamate receptors. The present experiments were performed to characterize the binding of $[^3H]$L-glutamate to membranes prepared from cockroach CNS. The assay was performed at 4°C in Tris-citrate buffer and was terminated by centrifugation.

Apparent values for K_D (0.83 ± 0.35 μM, n=5) and B_{max} of (20.5 ± 10.8 pmole of glutamate bound per mg of membrane protein, n=6) compare well with previous measurements obtained for the housefly (brain and thoracic muscle) and cockroach (nerve cord) by a filtration method[3]. Under the conditions adopted here binding was unaffected by Ca^{2+} or Cl^-. Analysis of the data with the Scatchard and Hill approaches suggests a single binding site with a Hill coefficient of 0.98 ±0.03 (n=6).

Several inhibitors of glutamate binding were tested at 1.0 mM concentration. The relative potency of inhibitors at this concentration was ibotenate > quisqualate > L-aspartate > L-APB > kainic > NMDA > D-APV.

The dissociation constant (K_D), and pharmacological profile of this site are consistent with binding to a putative glutamate receptor.

Kinetic studies were conducted using freeze-dried membranes and similar values for K_D and B_{max} were obtained. Such freeze-dried membrane samples were subjected to increasing doses of ionizing radiation. The relationship between functional inactivation and radiation dose is a function of molecular target size[4]. The estimated molecular weight for the putative L-glutamate receptor is 75.300 ± 5,600 (n=4) daltons (see Figure 1).

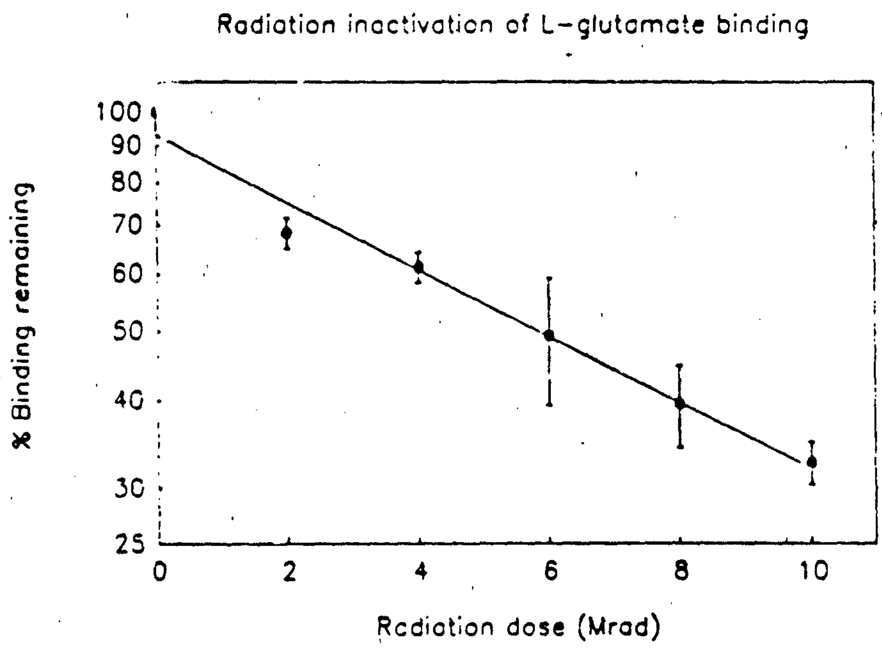


Figure 1. Effects of irradiation on the specific binding of [³H]L-glutamate to a cockroach CNS membrane preparation. Specific binding is defined as that displaceable by excess unlabelled L-glutamate (1.0 mM) in the presence of 50 nM [³H]L-glutamate. Bars represent twice the standard error of the mean (n=4).

References

1. Usherwood P. N. R. (1981) In DiChiara G., Gessa G. L. (eds) Glutamate as a neurotransmitter, pp. 183-193, Raven Press, New York.

2. Wafford K. A. and Sattelle D. B. (1986) Neurosci. Lett. 63:135-140

3. Sherby S. M., Eldefrawi M. E., Wafford K. A., Sattelle D. B. and Eldefrawi A. T. (1987) Comp. Biochem. Physiol. 87C:99-106.

4. Kepner G. R. and Macey R. I. (1968) Biochim. Biophys. Acta 163:188-203.

THE CONVULSANT RECOGNITION SITE OF THE GABA RECEPTOR COMPLEX IN TERATOGENICITY OF POLYCHLOROCYCLOALKANE INSECTICIDES IN CHICKEN EMBRYOS

Josef Seifert

Department of Agricultural Biochemistry, University of Hawaii, Honolulu, Hawaii 96822, U.S.A.

This paper considers the interaction of polychlorocycloalkane insecticides with the convulsant [picrotoxinin or t-butylbicyclophosphorothionate (TBPS)] recognition site of the GABA receptor complex as the mechanism of their teratogenicity in chicken embryo.

Ontogenesis of the convulsant recognition site in embryonic chicken brain was defined with the radioligands [^{35}S]TBPS and [^{3}H]t-butylbicycloorthobenzoate ([^{3}H]TBOB). Binding of the radioligands is detectable from day 6 of incubation. The increase in binding capacity during embryogenesis and after hatching is due to an increase in the number of binding sites with no change in affinity of the recognition site for TBOB. The embryonic convulsant recognition site is pharmacologically equivalent to that of mature brain based on its affinity to [^{3}H]TBOB, association and dissociation rates of the radioligand from the receptor, regulation and modulation by GABA, and inhibition of [^{3}H]TBOB binding to brain membranes by the GABA antagonists endrin, picrotoxinin and TBPS (Fig.1). Early formation of the convulsant recognition site and its susceptibility to GABA antagonists makes the embryo a possible target for drugs and environmental toxicants which act at this site in adult organisms.

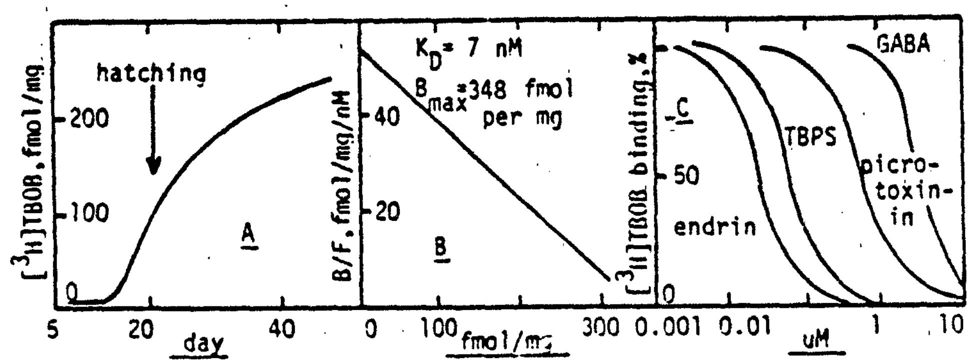


Fig. 1. Ontogenesis and properties of the convulsant recognition site in chicken embryos and chicks. EDTA/water-dialyzed brain membranes prepared from 17-day old embryos and 10-day old chicks were used to investigate ontogenesis of the convulsant recognition site measured by [^{3}H]TBOB binding (A), Scatchard analysis (embryo)(B), and inhibition of [^{3}H]TBOB binding to brain membranes (C).

Polychlorocycloalkane insecticides were examined for teratogenicity in chicken embryos. Endrin was the most potent teratogen causing cerebrocervical edema and arthrogryposis at doses 0.5-3.0 mg/egg as specific defects when injected into fertile chicken eggs between day 4 and 10 of incubation. Four lines of evidence suggesting involvement of the embryonic convulsant recognition site in teratogenicity of endrin were obtained by investigating the effects of other GABA antagonists and/or protective agents. First, GABA antagonists such as picrotoxinin, which acts at the convulsant recognition site[2], and 3-mercaptopropionic acid, which inhibits glutamate decarboxylase-catalyzed biosynthesis of GABA[3], induce the same malformation in chicken embryos as endrin. Second, the malformations induced by endrin are alleviated and prevented by phenobarbital and diazepam, antidotes of acute poisoning by convulsive toxicants[4]. Third, the teratogenicity of polychlorocycloalkane insecticides correlates with their mammalian toxicity and in vitro ability to decrease the radioligand binding to rat brain membrane preparations. Finally, the severity of malformations induced by endrin in chicken embryos correlates with a decrease in [^{3}H]TBOB binding to chicken embryonic brain membranes.

This study provides evidence about the regulatory role of the GABA (pro)receptor complex in early embryogenesis. Blockade of this system by convulsive GABA antagonists results in the malformations arthrogryposis and cerebrocervical edema in chicken embryos.

1. Squires, R.F., Casida, J.E., Richardson, M. and Saederup, E. Mol. Pharmacol. 1983, 23 326-336.
2. Ticku, M.K., Ban M. and Olsen, R.W. Mol. Pharmacol. 1978, 14 391-402.
3. Loscher, W. Biochem. Pharmacol. 1979, 28 1397-1407.
4. Gossel, T.A. and Bricker, J.D. in "Principles of Clinical Toxicology" 1984, p.137 (Raven Press).

Binding of Glutamate to a Membrane Fraction from Locust (*Schistocerca grgaria*) Central Nervous System.

George Usoh, Ian R. Duce and Peter N. R. Usherwood

Department of Zoology, University of Nottingham, University Park, Nottingham NG72RD, England.

The supra-oesophageal and sub-oesophageal ganglia were removed from the heads of 100 adult locusts and placed in Tris acetate buffer (5mM) Ph7.0 at $0^{o}C$. The ganglia were frozen for 1 hour at $-80^{o}C$ and then thawed prior to suspension in 120ml of Tris acetate buffer. The material was homogenised and centrifuged for 20 mins at 70,000 x g. The pellet was resuspended and filtered to remove coarse material. The suspension was centrifuged again and the pellet resuspended in fresh buffer. This procedure was repeated four times with a freeze/thaw step after every two centritugations in order to remove all traces of endogenous L-glutamate. The final pellet was suspended in Tris acetate buffer (50mM) at a concentration of 50 CNS per ml. This material was either used immediately or stored at $-80^{o}C$ for subsequent use.

^{3}H L-glutamate (20nM) with and without various concentrations of unlabelled ligands was incubated with 40ul of CNS membrane suspension in a total volume of 150ul. At the end of the incubation period bound L-glutamate was separated from unbound by one of two methods: The mixture was centrifuged at 15,000 x g for 30 secs, the supernatant was removed by aspiration and the pellet was brietly washed. The pellet was then solubilised and the radiaoctivity determined by liquid scintillation counting. Alternatively the incubation mixture was vacuum filtered through GF/B (Whatman) glass fibre filters. The filters were washed once with 7.5ml of 5mM Tris acetate buffer and the retained radioactivity was determined as above. Non-specific binding was determined by measuring the amount of binding retained in the presence of 10μM unlabelled L-glutamate. The protein concentration of the membrane preparation was determined using Peterson's modification of Lowry's method.

The time course of binding and dissociation of L-glutamate was rapid, reaching a maximum within 5 mins. More than 90% of this binding could be dissociated within 5 minutes by adding excess L-glutamate The remainder was dissociated within a further ten mins. With the tiltration assay Scatchard analysis of the binding suggested the presence of two saturable binding sites, a high attinity binding site with a K_d of 20nM and a B_{max} of 0.8pmol/mg, and a lower affinity site with a K_d of 230nm and a B_{max} of 5pmol/mg (Fig.1). With the centrifugation assay two binding sites were also present. The high affinity binding site had a similar affinity, but the value for B_{max} was 2pmol/mg. However the lower affinity site appeared to be unsaturable. In both assays, at low L-glutamate concentrations (<10nm), binding showed positive cooperativity.

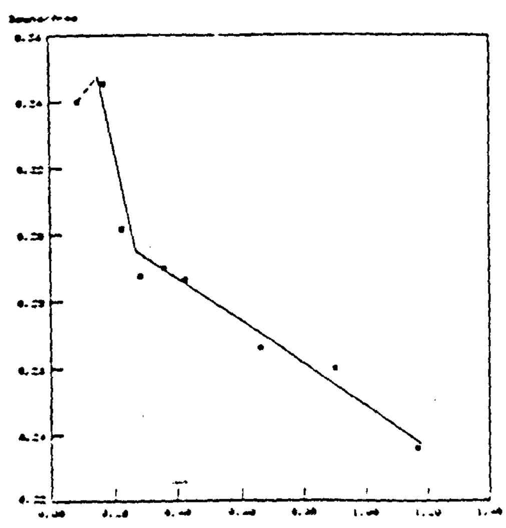
Several ligands which have been reported to bind to L-glutamate receptors were examined as unlabelled competitors for ^3H L-glutamate binding. Quisqualate, kainate, NMDA, domoate, ABPA and a kainate analogue cis 2,3,-PDA bound to the high affinity site with an apparent K_d of 15-20nM. A comparison was made of the ability of various ligands to displace binding of 20nM L-glutamate. The results are shown in Table 1.

This work was supported by an AFRC grant to PNRU and IRD

Table 1. % Reduction in binding of 20nM ^3H L-glutamate to Locust CNS membranes in the presence of the following compounds (8μM).

	Data from Centrifugation assay	Data from Filtration assay
L-glutamate	100	100
Ibotenate	60-80	90
L-GDEE		
L-GME		
D-glutamate	}30	}70
Chlorpromazine		
L-Quisqualate		
ABPA		
cis-2,3-PDA	}35	}25
APV		
Glu-hydroxamate		
NMDA		
Domoate	}25	}15
Kainate		
d-Quisqualate		
Dihydro-kainate		
-keto-kainate		
L-aspartate		
D-aspartate		
AMPA		
TPMP	}0-10	}<10
Ketamine		
Chlorisondamine		
GABA		
MLV-5869		
MLV-69.A		

Fig 1 Scatchard analysis of L-glutamate binding to locust CNS membranes using a filtration assay.



Binding of Phencyclidine to a Membrane Fraction from Locust (*Schistocerca grgaria*) Central Nervous System.

George Usoh, Ian R. Duce and Peter N. R. Usherwood

Department of Zoology, University of Nottingham, University Park, Nottingham NG72RD, England.

Phencyclidine (PCP) is a dissociative anaesthetic which is thought to bind to at least two sites in vertebrate central nervous system (CNS)[1], called the PCP site and the sigma site. The PCP site is thought to be associated with glutamate receptor ion channels and it is at this site that PCP is thought non-competitively to antagonise n-methyl-D-aspartate reponses [2]. PCP is also a non-competitive antagonist of the quisqualate sensitive receptor of locust leg muscle (Usherwood unpublished data). This compound possesses a number of chemical features which might be of value in the isolation and purification of glutamate receptors, both peripheral and central in insects. We present here results which demonstrate high affinity binding of PCP to locust CNS membranes.

The heads of 100 adult locusts were removed. The supra-oesophageal and sub-oesophageal ganglia were dissected out and placed in Tris acetate buffer (5mM) at 0°C. The ganglia were frozen for 1 hour at -80°C and then thawed prior to suspension in 120ml of Tris acetate buffer. The material was homogenised and centrifuged for 20 mins at 70,000 x g. The pellet was resuspended and filtered to remove coarse material. The suspension was centrifuged again and the pellet resuspended in fresh buffer. This procedure was repeated four times with a freeze/thaw step after every two centrifugations. The final pellet was suspended in Tris acetate buffer (50mM) at a concentration of 50 heads per ml. This material was either used immediately or stored at -80°C for subsequent use.

^{3}H phencyclidine(PCP) (5nM) with and without various concentrations of unlabelled ligands was incubated in a total volume of 150µl with 40µl of CNS membrane suspension at 30 °C. At the end of the incubation period bound PCP was separated from unbound PCP by vacuum filtration through GF/B (Whatman) glass fibre filters. (These filters were rapidly soaked in buffer containing 0.2% polyethylene glycol prior to use). The filters were washed once with 10 mls of 5mM Tris acetate buffer and the retained radioactivity was determined by liquid scintillation spectroscopy. Non-specific binding was determined by measuring the amount of binding retained in the presence of 10µM unlabelled PCP. Protein concentration of the membrane preparation was determined using Peterson's modification of Lowry's method.

The binding of PCP reached a maximum after 40 mins with a time to half maximal binding ($t_{1/2}$) of 10 mins. Addition of (1µM) PCP resulted in complete dissociation of the bound PCP with a $t_{1/2}$ of 20 mins. The binding of PCP was highly temperature dependent with a maximum at 30 °C, the incubation temperature used in most

experiments.

Scatchard analysis of PCP binding revealed only one binding site with a K_d of 11nM and a B_{max} of 2.6pmol/mg. The pharmacology of the phencyclidine binding site was examined by deriving IC_{50} values for a number of compounds which have been reported to act as non-competitive antagonists of glutamate and other receptors (Table 1).

Several ligands for the glutamate receptor were also examined as competitors for PCP binding sites by examining their potency in displacing 5nM ^{3}HPCP. At concentrations up to 1uM no displacement was produced by: L-glutamate, L-aspartate, D-aspartate, ibotenate, kainate, quisqualate, kanamycin, NMDA, or glycine.

This work was supported by an AFRC grant to PNRU and IRD.

Table 1. Effect of various compounds on ^{3}H PCP binding to locust CNS membranes. Data are presented as IC_{50} values (µM) required to displace 5nM PCP.

TCP	0.01
Phencyclidine	0.05
TPMP	0.16
MLV6976	1
$Argiotoxin_{623}$	1
Spermine	25
Ketamine	125
Chlorisondamine	180
Streptomycin	2000

1.Sonders, M.S. Keana, J.F.W. and Weber, E. TINS 11 37-40 (1988).

2. Anis, N. Berry, S.C. Burton, N.R. and Lodge, D. Br. J. Pharmacol. 79 565-575 (1983).

CHARACTERISATION OF SPECIFIC BINDING SITES FOR (^{3}H) - SAXITOXIN IN THE CATERPILLAR CNS.

Bernard Verdon, Stuart E. Reynolds and George G Lunt

School of Biological Sciences, University of Bath, Claverton Down, Bath England BA2 7AY.

Saxitoxin (STX) is a heterocyclic guanidine toxin that binds to the vertebrate action potential sodium channel and renders it impermeable to sodium ions. It acts at the same site as tetrodotoxin (TTX)[1]. Sodium channels are thought to be the principal target of DDT and pyrethroid insecticides, perhaps acting at exposed neuromuscular junctions to promote neurotransmitter release. This action of pyrethroids is abolished by TTX[2]. It was therefore of interest to characterise the binding of (^{3}H)-STX to an insect nervous system and its interaction with pyrethroid insecticides.

Binding experiments utilised simple homogenates of the entire CNS of fifth instar larvae of the tobacco hornworm, Manduca sexta, which were used without further fractionation suspended in 50 mM sodium phosphate buffer, pH 7.4, at a protein concentration of approx. 0.2 - 0.3 mg ml^{-1}. (^{3}H)-STX (68 Ci mmol^{-1}) was from Amersham. Binding was initiated by the addition of (^{3}H)-STX to 100 µl aliquots of homogenate, which were kept on ice throughout the experiment. After the incubation period (15 - 20 min, except for kinetic experiments) bound (^{3}H)-STX was separated from the free ligand by rapid dilution and vacuum filtration on Whatman GF/C filters presoaked in 0.3% polyethylenimine. Non-specific binding was determined in parallel experiments where 1 µM TTX was included in the buffer. Association kinetics were determined by varying the length of the incubation. Dissociation was followed by determining specific binding at varying intervals after 100-fold dilution of a pre-equilibrated aliquot.

The caterpillar CNS contained high-affinity, saturable (^{3}H)-STX binding sites. Specific binding was more than 95% of the total. Scatchard analysis was consistent with a single class of binding sites giving an estimated K_D = 1.53 + 0.29 nM (mean + SD, 3 experiments) and B max = 3.41 + 0.91 pmol per mg protein. Hill plots were linear and had slopes close to 1.0 indicating lack of cooperativity between binding sites. Determination of association and dissociation kinetics revealed that both on - and off - reactions were rapid (< 2 min). Estimation of K_D from kinetic data gave a figure of 0.70 nM, in reasonable agreement with the Scatchard values. Specifically bound (^{3}H)-STX was displaced by TTX with an IC_{50} = 0.68 nM. No significant displacement of (^{3}H)-STX binding was observed when the CNS homogenates were pretreated (30 min) with DDT, cypermethrin, deltamethrin, fenpropathrin, permethrin or tetramethrin, all at 10 µM. It was necessary to include 4% acetone in the buffer to solubilise these compounds at this concentration. Binding of (^{3}H)-STX was unaffected in controls that contained 4% acetone alone.

We conclude that binding of (^{3}H)-STX to *Manduca* CNS has similar characteristics to that seen in CNS of vertebrates[3] and of the two other insects that have previously been investigated - the locust *Locusta migratoria*[4] and the fly *Drosophila melanogaster*[5]. Thus only one class of binding site was observed, which had a K_D in the nanomolar range.

Vertebrates have sodium channels in tissues other than nerve, eg skeletal muscle. Homogenates of *Manduca* body wall muscle and of salivary glands did not bind significant amounts of (^{3}H)-STX) suggesting that sodium channels are restricted to the CNS. This is in accord with electrophysiological evidence that insect muscles do not have sodium channels[6].

Within the CNS, distribution of (^{3}H)-STX binding sites was uniform. Thus brain, other ganglia, and connectives all had a similar density of binding sites. Apparent B_{max} values were 2.95 (brain), 2.36 (ganglia), and 3.10 pmol per mg protein (connectives). This density of (^{3}H)-STX binding sites is high for a crude homogenate, being comparable with that seen in membrane preparations from mammalian brain (eg[7]).

Presumably the (^{3}H)-STX binding site defines a functional sodium channel in the caterpillar CNS. The lack of direct interference between DDT, pyrethroids and (^{3}H)-STX binding indicates that these insecticides disrupt sodium channel function at a site that is distinct from and does not interact with the (^{3}H)-STX binding site.

References

1. Catterall, W.A. *Science* 1984, **223** 653-661.

2. Salgado, V.L., Irving, S.N., Miller, T.A. *Pestic. Biochem. Physiol.* 1983, **20**, 100-114.

3. Weiland, G.A., Molinoff, P.B. *Life Sciences* 1981, **29**, 313-330.

4. Gordon, D., Zlotkin, E., Catterall, W.A. *Biochim. Biophys. Acta* 1985, **821**, 130-136.

5. Gitschier, J., Strichartz, G.R., Hall, L.M. *Biochim. Biophys. Acta* 1980, **595**, 291-303.

6. Ashcroft, F.M., Stanfield, P.R. *J. Physiol.* 1982, **323**, 93-115.

7. Weigele, J.B., Barchi, R.L. *FEBS Lett.* 1978, **91**, 310-314.

A MOLECULAR MODELLING APPROACH TO THE UNDERSTANDING OF PYRETHROID ACTIVITY

Jette R. Byberg and Flemming S. Jørgensen
Department of Chemistry BC, Royal Danish School of Pharmacy,
Universitetsparken 2, DK-2100 Copenhagen, Denmark

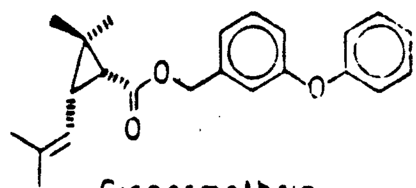
Per D. Klemmensen
A/S Cheminova, P.O. Box 9, DK-7620 Lemvig, Denmark

The pyrethroids are highly valuable insecticides as they combine high insecticid activity with low mammalian toxicity. Nearly all of the compounds contain chiral centers and in most cases only one of the stereoisomers are active, thus indicating a receptor-mediated mechanism of action.

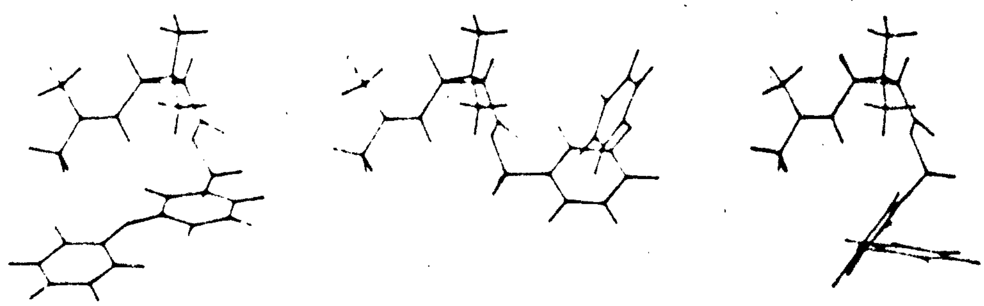
In order to identify the essential conformation(s) of the active molecules, nine potent and structurally different type I pyrethroids have been selected for analysis. For these compounds the three-dimensional arrangement of the groups essential for biological activity (a pharmacophore model) has been identified for the acid and alcohol moieties, respectively, and the active conformation of the acid and alcohol moieties deduced.[1]

For each of the esters the acid and alcohol moieties in their active conformations were combined to the original esters and the complete molecules evaluated. Subsequent energy minimization combined with geometrical overlapping of relevant low energy conformations, yielded three possible active conformations for each pyrethroid ester. The three pharmacophore models thus obtained turned out to be equally reasonable.

Below the three possible active conformations are illustrated for one of the nine pyrethroids.



Cispermethrin



The different conformations of the pyrethroid esters have been evaluated by different computational approaches and by comparison with experimentally determined molecular structures extracted from the Cambridge Structural Database. Finally, conformations of other known pyrethroid compounds (active as well as inactive) have been compared with the three pharmacophore models in order to distinguish between these models.

Determination of the essential conformation for the active molecules may help one to infer the complimentary features of the receptor and hopefully be useful in the design of novel pyrethroids.

1. Byberg, J.R., Jørgensen, F.S. and Klemmensen, P.D., J. Comp.-Aid. Mol. Des. 1987 1 xxx-xxx.

THE USE OF MICROCOMPUTER BASED DATA LOGGING AND ANALYSIS SYSTEMS IN NEUROTOXICOLOGICAL STUDIES OF PESTICIDE ACTION

Raymond Crichton, Richard Greenwood, Martyn G. Ford and David W. Salt.[a]

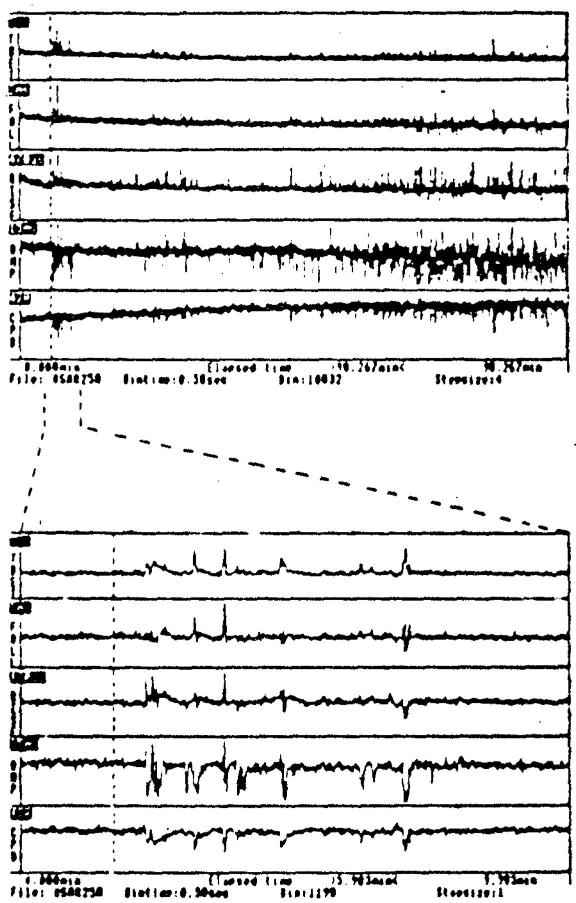
School of Biological Sciences, Portsmouth Polytechnic, Portsmouth, England PO1 2DY
[a] Department of Mathematics and Statistics.

INTRODUCTION

In studies of the action of neurotoxic materials, a number of properties of the nerve are affected, producing changes in the behaviour of the nerve. An array of symptoms, including changes in frequency, shape and pattern of firing of action potentials (APs) may be observed. These responses may vary with concentration of the applied toxicant, and the elapsed time following application of the poison.

The length of an experiment can vary from minutes to hours. The amount of information gathered in an analogue recording may be considerable, especially where long elapsed times are involved. Using conventional recording techniques, such as pen and UV recorders, it becomes an enormous and daunting task to analyse and measure responses of interest. The use of computers to log and analyse these data makes it possible to summarise the results of even long experiments, and facilitates the analysis of trends in the data.

In pyrethroid poisoned nerves, a number of changes in activity may occur[1]. These may be observed in isolated nerve preparations, where the dose of pesticide is applied in the bathing medium, or in whole insect preparations, where it may be applied topically or by injection. In all cases there will be a finite elapsed time before the pesticide reaches the site of action in sufficient amount to cause symptoms of poisoning. Depending on the applied dose, and properties of the test material, the initial delay is followed by a change, usually an increase, in frequency of APs. This may be followed in turn by bursting of APs and/or eventually nerve block. We report a computer based method which enables the course of poisoning by neurotoxicants such as pyrethroids to be followed routinely. The approach shows promise for use in both fundemental studies, and laboratory screening of novel materials.



KEY TBET = time (ms) between action potentials (APs): FALL = mean fall time (ms) of APs: RISE = mean rise time (us) of APs: AMP = mean amplitude (mV) of APs: CPB = number of APs per bin. The vertical, broken line indicates the time of application of pesticide.

Figure 1. A summary of the time course of pyrethroid (QSAR25) poisoning in an isolated, housefly haltere nerve preparation. In figure 1a, every fourth bin (0.5s) was sampled over 90.267 minutes. In figure 1b, a small section of the record in figure 1a was expanded by sampling every bin (0.5s) over 5.983 minutes.

In order to data log changes in transmembrane electrical potential in nerve cells, time is considered as a series of discrete intervals (bins). The number of APs which exceed a user defined threshold voltage in each bin is counted, and the amplitudes, rise times, and fall times of APs summed within each bin. From these data, the average amplitude, rise time, fall time, and time between APs is calculated for each bin. Using this approach, neurophysiological data can be monitored over long periods (figure 1).

An application which illustrates the utility of this data logging technique is described below. The results summarised in figure 1 were obtained from neurophysiological recordings made following exposure of an isolated, predominantly sensory, housefly haltere nerve preparation[2,3] to a 10^{-8} molar suspension of a rapidly acting pyrethroid, QSAR25 (4-methylsulphonylbenzyl-(1*RS*)-*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate), in insect Ringer. The course of events, as described earlier, commonly observed in pyrethroid poisoned nerves, was seen with this compound. However, data logging revealed an unusual feature at early elapsed times. Over the first five minutes following exposure, there was a reduction in the frequency of APs. As the course of poisoning proceeded, a more typical response pattern developed, with an increasing frequency of APs and increasing variation in numbers of APs per bin, mean amplitude, and mean rise and fall times. The increasing variation may reflect the recruitment of neurones as pesticide distribution proceeded within the preparation.

This technique may be useful in studying the relationship between pharmacokinetics and the course of neurotoxic symptoms in whole animals. More importantly, it is potentially useful for the automatic screening of candidate insecticides on isolated preparations, and on restrained, whole insects.

1. Gammon, D.W., Brown, M.A., Casida, J.E. Pestic. Biochem. Physiol. 1981 15 181-191

2. Sandeman, D.C., Markl, H. J. exp. Biol. 1980 85 43-60

3. Burt, P.E., Goodchild, R.E. Rothamsted Experimental Research Station Report 1981, pp. 127-128

TOXICITY AND DISTRIBUTION OF AVERMECTIN B_1 IN LARVAE OF <u>SPODOPTERA LITTORALIS</u> AND <u>HELIOTHIS ARMIGERA</u> (LEPIDOPTERA: NOCTUIDAE).

Paul T. Christie and Denis J. Wright.

Department of Pure and Applied Biology, Imperial College
Silwood Park, Ascot, Berkshire, U.K. SL5 7PY.

The toxicity of avermectin B_1 (AVM) has been shown to differ considerably among lepidoptera[1]. In the present study, marked changes in the relative toxicity of topically-applied AVM (ng/mg) were also found to occur between the second and sixth larval instars of <u>Spodoptera littoralis</u>. The activity of AVM decreased with increasing larval instar up to the fifth instar but increased 386 fold in the sixth instar. In contrast, the toxicity of AVM decreased slightly from the fifth to the sixth larval instar of <u>Heliothis armigera</u>.

The toxicity of AVM against fifth but not sixth larval instars of <u>S.littoralis</u> was considerably increased by injection of the pesticide or by additional treatment of the larvae with the mixed function oxidase (MFO) inhibitor piperonyl butoxide (PB). PB had no significant effect on the toxicity of AVM against the fifth larval instar of <u>H.armigera</u>.

Concurrent work has related radioactivity in the nervous system of <u>S.littoralis</u> and <u>H.armigera</u> larvae, following topical application of 3H-AVM, with symptoms of poisoning.

The results suggest that the relative insensitivity of the fifth compared with the sixth larval instar of <u>S.littoralis</u> to AVM may be accounted for in terms of reduced penetration and enhanced metabolism. Studies on the action of AVM on the nervous system of <u>S.littoralis</u> and <u>H.armigera</u> are in progress.

1. Anderson, T.E., Babu, J.R., Dybas, R.A. and Mehta, H. J. [illegible] 1986 79 197-201.

PYRETHROID-INDUCED ALTERATIONS IN MAMMALIAN SYNAPTIC FUNCTION

Janis T. Eells

Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, WI 53226 U.S.A

The synthetic pyrethroids are potent and widely used neurotoxic insecticides. These agents have been divided into two major classes on the basis of their structures and neurophysiological actions.[1] Pyrethroids containing an α-cyano substituent have been classified as type II pyrethroids. In insect nerve preparations, type II pyrethroids have been shown to depolarize presynaptic nerve terminals resulting in an increase in spontaneous neurotransmitter release and a blockade of synaptic transmission.[2] We have recently shown that type II pyrethroids increase spontaneous neurotransmitter release in mammalian brain slices.[3,4] The purpose of this investigation was to determine the action of type II pyrethroids on nerve membrane potential and synaptic transmission in mammalian brain.

The accumulation of ^{3}H-tetraphenylphosphonium (TPP) was used to estimate membrane potential in rat forebrain synaptosomes. The type II pyrethroid, deltamethrin (DM) enhanced membrane depolarization induced by sodium channel activation (veratridine, 30 μM) or by increasing extracellular potassium concentration (20 mM). The enhancement of veratridine-dependent depolarization by DM was further shown to be concentration dependent (10 nM-10 μM) and tetrodotoxin sensitive. DM had no effect on membrane potential in the absence of a depolarizing stimulus indicating that pyrethroids do not depolarize mammalian synaptosomal membranes at resting membrane potential. These data support the hypothesis that pyrethroids stabilize the activated voltage sensitive sodium channel in an open conformation prolonging sodium conductance and increasing membrane depolarization.

The actions of DM on synaptic transmission were studied in slices of guinea pig olfactory cortex. Field potentials were recorded from the surface of the prepyriform cortex in response to stimulation of the lateral olfactory tract (LOT) with single or paired electrical pulses. The evoked potential (EP) consisted of a negative wave (1-3 mV amplitude, 4-6 msec latency) which primarily reflects excitatory postsynaptic potentials arising from superficial synapses activated by LOT fibers. DM significantly depressed the EP amplitude at low stimulation intensities (1-5 V). As stimulus intensity was increased (5-20 V), the response in pyrethroid-treated preparations increased to the same maximal response seen in controls indicating that DM elevated the stimulus threshold required to elicit a maximal response. When paired-pulse stimulation (20-100 msec interpulse interval) was employed, DM produced a frequency-dependent paired-pulse inhibition. The EP ratio (test EP/conditioning EP) after exposure to DM (10 μM) did not exceed 1.0 and was profoundly depressed at 20 and 40 msec intervals (0.44 and 0.69, respectively). In contrast, paired pulse facilitation was observed in control preparations with EP ratios between 1.1 - 1.25. These data demonstrate that deltamethrin reduces the synaptic input from the LOT to the cortical neurons. In non-mammalian nerve preparations, type II pyrethroids have been shown to depolarize the nerve membrane resulting in an inhibition of evoked activity and a frequency dependent conduction block.[1,2,5] Our data provide evidence that type II pyrethroids exert a similar action in the mammalian CNS. (Supported by NIH-BRSG Investigator Award and NIH ES-01985).

1. Narahashi, T. Neurotoxicol. 1985, 6: 3-32.
2. Salgado, V.L., Irving, S.N., Miller, T.A. Pest. Biochem. Physio. 1983, 20: 100-114.
3. Eells, J.T. and Dubocovich, M.L. Pestic. Sci. 1985, 16: 439-440.
4. Eells, J.T. and Dubocovich, M.L. J. Pharmacol. Exp. Ther. 1988, (in press).
5. Vijverberg, H.P.M. and de Weille, J.R. Neurotoxicol. 1985, 6: 23-34.

TIME-DEPENDENT INHIBITORS OF ACETYLCHOLINESTERASE

Derek Gammon*, Robert M. Kral, Donald A. Shaw,

FMC Corporation, Agricultural Chemicals Group, P.O.Box 8

Princeton, N.J. 08543, USA

A novel class of mechanism-based inhibitors of acetylcholinesterase will now be described. These compounds demonstrate rapid inhibition of the enzyme in vitro in which loss of activity is first order. Kinetic data suggest that the inhibitor is interacting with the enzyme at the active site. In vitro activity is comparable to the standards carbofuran and paraoxon. In vivo testing on electrode implanted cockroaches shows that these compounds induce repetitive firing which is characteristic of AChE inhibition.

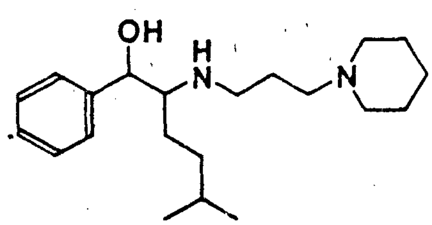
NEUROPHARMACOLOGICAL ACTIONS OF MLV-COMPOUNDS IN INVERTEBRATES AND VERTEBRATES

Y. GOTO, M. ISHIDA and H. SHINOZAKI

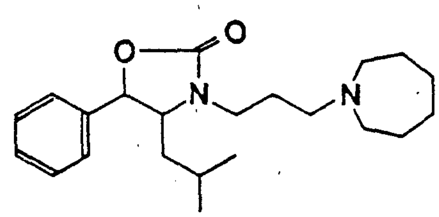
The Tokyo Metropolitan Institute of Medical Science, Tokyo 113, Japan

MLV-6976 (NC-1200) and MLV-5860 possess some interesting neuropharmacological actions in the crustacean neuromuscular junction and the mammalian CNS. One of the most striking actions of MLV-6976 was a potent inhibitory action on the mammalian CNS (Masaki and Shinozaki, Br. J. Pharmacol.,(1986), 89, 219-228), together with glutamate blocking actions. In order to clarify the mode of actions of centrally acting drugs, the crayfish NMJ has been relatively widely used because the junction provides an excellent model for studying the mechanism of action of drugs on synaptic transmission (Shinozaki, Prog. Neurobiol.,(1980), 14, 121-155). We report here on some neuropharmacological actions of MLV-6976 and its derivatives comparing their actions in the invertebrates with those in the vertebrates.

(1) Glutamate inhibitory actions of MLV-6976 by open channel block of the glutamate-gated channel at the crayfish NMJ: MLV-6976 reduced the amplitude of the glutamate response at the crayfish NMJ, but MLV-6976 was weaker than



MLV 5860



MLV 6976

MLV-5860 comparing the effective concentration on a molar basis. MLV-6976 (30 μM) decreased the repetitive synaptic current induced by iontophoretic glutamate pulses to about one third of its control amplitude within 2 min. This blocking action of MLV-6976 on the glutamate response was use dependent, suggesting that the action of MLV-6976 is explained by open channel block of the glutamate-gated channel at the crayfish NMJ.

(2) Glutamate blocking actions of MLV-6976 in the new-born rat spinal cord: When depolarizing responses to excitatory amino acids were extracellularly recorded from the new-born rat spinal cord ventral roots, responses to glutamate, NMDA, kainic acid and quisqualic acid were almost equally depressed by MLV-6976 (0.3 mM), suggesting that MLV-6976 did not block the responses of excitatory amino acids mediated by the specific glutamate receptor subtype. MLV-6976 also decreased the amplitude of the ACh response, but responses to glutamate were much more affected by MLV-6976 than the ACh response. Responses to Substance P was markedly reduced by MLV-6976, but actions of inhibitory transmitters such as GABA and glycine were not affected by the drug.

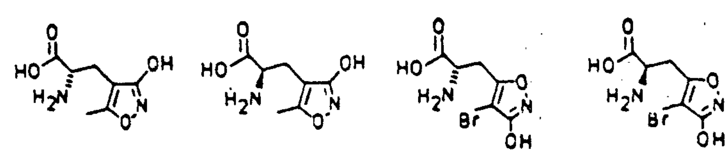
(3) Depression of rat decerebrate rigidity: MLV-6976 markedly decreased the severity of rat anemic decerebrate rigidity (Masaki and Shinozaki, Br. J. Pharmacol., (1986), 82, 523-531). When kynurenate, ketamine, MLV-6976, MK-801, CPP and APH were intravenously administered to decerebrate rats, they reduced the severity of decerebrate rigidity in a dose dependent manner, although there was a large variance in effective doses among them. At the present, there has been no evidence that the glutamate antagonist is directly related to the relief of decerebrate rigidity, however, these results provide a key to elucidation of the glutamate function in the mammalian CNS.

STEREOCHEMICAL ASPECTS OF GLUTAMIC ACID RECEPTOR AGONISTS

Jan J. Hansen, Birgitte Nielsen, Annemarie Reinhardt, Lotte Brehm and Povl Krogsgaard-Larsen

Department of Chemistry BC, The Royal Danish School of Pharmacy, 2 Universitetsparken, DK-2100 Copenhagen, Denmark

(*S*)-Glutamic acid (L-Glu) and possibly also (*S*)-aspartic acid (L-Asp) appear to be excitatory transmitters in the mammalian central nervous system (CNS). The existence of at least three receptor classes for excitatory amino acids in the CNS is fairly well established from electrophysiological *in vivo* studies and *in vitro* binding experiments. The three receptor classes, which eventually may be further subclassified, are termed NMDA, QUIS/AMPA and KAIN receptors. These receptors have different pharmacological profiles and seem to have different functions, membrane characteristics and localizations in the CNS. The QUIS/AMPA receptor and the NMDA receptor appear to be postsynaptic receptors participating in monosynaptic and polysynaptic transmission, respectively. Little is known about the physiological function of the KAIN receptor. The NMDA receptor shows a limited stereoselectivity for agonists and readily accepts the D-form of some agonists and the L-form of others. In contrast, antagonists at NMDA receptors are mainly of the D-form. No potent and selective antagonists at the QUIS/AMPA receptor are known, but agonists at this receptor are predominantly of the L-form.



	(S)-AMPA	(R)-AMPA	(S)-Br-HIBO	(R)-Br-HIBO
GLU Binding ($CaCl_2$)(IC_{50}, μM)	>100	>100	0.24	0.14
AMPA Binding (IC_{50}, μM)	0.030	0.76	0.34	22
Neuroexcitation (Rel. potency)	+++++	++(+)	++++	+

Ibotenic acid is a constituent of fly agaric, *Amanita muscaria*, and shows potent excitatory effects. Ibotenic acid mainly acts at NMDA receptors but also shows some affinity to QUIS/AMPA and KAIN receptors. By using ibotenic acid as a lead structure we have, however, developed a number of isoxazole amino acids with potent as well as selective excitatory actions at QUIS/AMPA and at NMDA receptors. These compounds were prepared by multi-step synthetic procedures as racemic compounds and have provided information about the structural and conformational requirements for activation of subtypes of central L-Glu receptors[1]. However, such structure activity studies of racemic compounds are of limited value unless followed by studies of the individual stereoisomers. We have therefore engaged in a systematic characterization of the enantiomers of isoxazole amino acids as obtained by resolution using enzymatic and chromatographic procedures. The isoxazole amino acids, (*RS*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and (*RS*)-α-amino-4-bromo-3-hydroxy-5-isoxazolepropionic acid (4-bromohomoibotenic acid, Br-HIBO), are potent and selective agonists at QUIS/AMPA receptors *in vivo* and *in vitro* without effect on NMDA receptors *in vivo* and KAIN receptors *in vitro*. The enantiomers of these two compounds have been prepared by enzymatic resolution[2,3] and show optical purities in excess of 99.5 % as determined by HPLC on a chiral stationary phase. The absolute configurations are based on the generally accepted stereoselectivity of the enzymes but has in the case of Br-HIBO been confirmed by X-ray crystallography. For both AMPA and Br-HIBO the excitatory effect mainly resides with the (*S*)-enantiomers (Figure). Similarly, the inhibitory effect on ^{3}H-AMPA binding is predominantly caused by the (*S*)-forms of these compounds. However, the effects on $CaCl_2$ dependent ^{3}H-L-Glu binding are different for the two compounds. In contrast to the (*S*)- and (*R*)-forms of AMPA, which are inactive, the enantiomers of Br-HIBO are (equi)potent inhibitors in this binding assay. These stereostructure activity studies are presently being extended to other isoxazole amino acids.

1. Hansen, J.J., Madsen, U., Nielsen, E.Ø., Nielsen, B., Brehm, L. and Krogsgaard-Larsen, P. in "New Methods in Drug Research", Vol. 3 (Makriyannis, A., ed.), Prous Science Publishers, Barcelona, Spain, 1988, in press.

2. Hansen, J.J., Lauridsen, J., Nielsen, E.Ø. and Krogsgaard-Larsen, P., *J. Med. Chem.* 1983, 26 901-904.

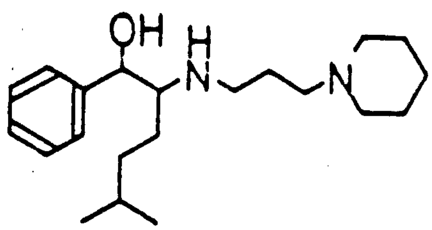
3. Nielsen, B., Hansen, J.J., Brehm, L., Nielsen, E. Ø., Krogsgaard-Larsen, P. and Curtis, D.R., manuscript in preparation.

NEW POTENT USE-DEPENDENT GLUTAMATE BLOCKERS AT THE CRAYFISH NEUROMUSCULAR JUNCTION.

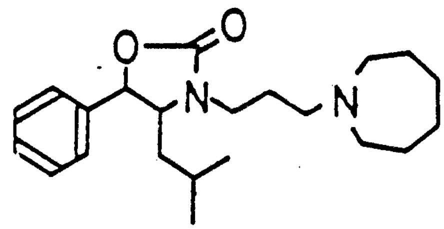
K. Hashimoto, H. Takeda, M. Masaki, and H. Shinozaki*
Research Laboratories of Nippon Chemiphar Co. Ltd., 1-22-1, Hikokawato, Misato-shi, Saitama 341, and *The Tokyo Metropolitan Institute of Medical Science, 3-18-22, Honkomagome, Bunkyo-ku, Tokyo 113, Japan.

MLV-5860 (1) and MLV-6976 (2) are trimethylenediamine derivatives which have been proved to possess a use-dependent glutamate blocking action in vertebrates and invertebrates (Shinozaki and Ishida, Brain Research, 372 (1986) 260-268; Masaki and Shinozaki, Brit. J. Pharmacol., 89 (1986) 219-228). In the present paper we describe the result of the examination on the structure-activity relationship of these compounds at the crayfish neuromuscular junction.

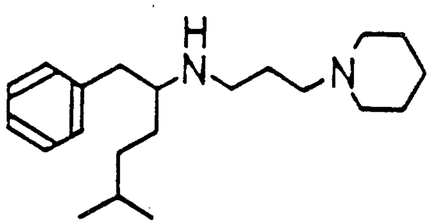
Most derivatives of MLV-5860 and MLV-6976, including some derivatives with functional groups such as ethoxycarbonyl, cyano and acetyl groups, did not demonstrate a more potent inhibitory activity than the lead compound, MLV-5860, on the glutamte response at the crayfish neuromuscular junction. However, the dehydroxylated derivative of MLV-5860 (3) demonstrated a powerful inhibitory action and its potency to block the glutamate response was almost equivalent to that of MLV-5860. These results suggest that the functional groups may not function to augment the glutamate blocking activity. On the basis of the result we obtained some potent blockers, modifying the skeletone of the compound (3). One of the most potent compounds is 1-[3-[4-methyl-1-(3-methylbutyl)pentylamino]propyl]piperidine (4).



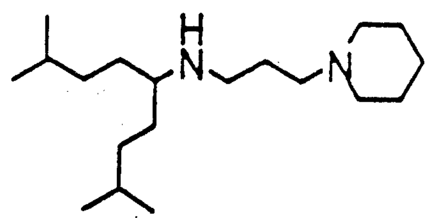
1



2



3



4

EFFECTS OF MLV-5860 AND MLV-6976 ON GLUTAMATE POTENTIALS AND GLUTAMATE UPTAKE IN LOCUST MUSCLE

Henk Karst, Albert Lind, Jan van Marle*, Tom Piek, Haruhiko Shinozaki⁺ and Janneke van Weeren-Kramer.

Departments of Pharmacology and Electronmicroscopy*, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands, and the ⁺Tokyo Metropolitan Inst. of Med.Sc., Bunkyo-ku, Tokyo 113, Japan.

Recently, as a result of screening a number of glutamate blockers the oxazolidinones MLV-5860 and MLV-6976 were found to reduce rigidity of decerebrate rats[1]. MLV-5860 was also found to be an inhibitor at the crayfish neuromuscular junction[2,3]. MLV-5860 (2×10^{-5} M) reduced the amplitude of repetitively-induced glutamate potentials in a time- and activity-dependent manner[3].

We have tested both toxins on the excitatory neuromuscular synaps of the locust (Schistocerca gregaria) skeletal muscle at two different ways: (1) studying the iontophoretically evoked glutamate potential, using muscles pretreated by concanavalin A (2×10^{-6} M), and (2) using EM-autoradiography to study the high affinity glutamate uptake[4].

At a concentration of $1.3\text{-}2.7 \times 10^{-6}$ M MLV-5860 reversibly blocks glutamate potentials to a steady state level of about 50-10% respectively (Fig. 1). The speed of reversibility is higher than with δ-PTX and much higher than with the spider toxins (this meeting). At a concentration of 5×10^{-5} M MLV-6976 reversibly blocks glutamate potentials to a steady state level of about 60% (Fig.1). At a high concentration of 200 µM both MLV-compounds did not change the glutamate uptake by terminal axons or glial cells.

References

1. Masaki, M.and Shinozaki, H., Br.J.Pharmacol, 1986, 89, 219-228.
2. Masaki, M., Moritoh, N., Hashimoto, K. and Shinozaki, H.

J.Pharm.Dyn.1985, 8, S-173.

3. Shinozaki, H. and Ishida, M. Brain Res. 1986, 372, 260-268.

4. Van Marle, J., Piek, T., Lind, A. and Van Weeren-Kramer, J. Comp. Biochem.Physiol. 1983, 74C, 191-194.

Fig. 1- Effects of MLV-5860, 2.7 x 10^{-8} M (A) and 1.3 x 10^{-8} M (B-D) and MLV-6976, 5.2 x 10^{-8} M (E-H) on glutamate potentials of locust muscle fibres pretreated with 2 x 10^{-6} M con A. Exponential decay has comparable time constants in control and after addition of the toxins (D and H). Arrows in A and E indicate the presence of the toxin in the bathing saline.

NEUROTOXIC ACTIONS OF A LIPID AMIDE ON THE COCKROACH NERVE CORD AND ON LOCUST SOMATA MAINTAINED IN SHORT-TERM CULTURE: A NOVEL PREPARATION FOR THE STUDY OF Na^+ CHANNEL PHARMACOLOGY.

George Lees and Paul E. Burt

Pesticide Research Department, The Wellcome Foundation Ltd, Berkhamsted, Herts, UK.

In previous investigations of the neurotoxic action of lipid amides[1] the occurrence of large, TTX-sensitive, depolarising after-potentials following axonic spikes suggested an action on sodium gates in neurones. In subsequent experiments, again with mannitol-gapped whole cockroach abdominal nerve cords, potassium conductances were suppressed by perfusion with cockroach saline containing 25mM TEA chloride, 10mM 4-AP and 3.1mM CsCl. Electrical stimulation resulted in an action potential followed by a short (40-80 ms) depolarizing after-potential associated with a brief after discharge.

When the lipid amide (2E,4E)-N-isobutyl-11,11-difluoro-undeca-2,4,10-trienamide (I)was added to the perfusate at 10μM, large and prolonged (1.5-2.5 sec) depolarising after-potentials were superimposed. The compound action potential and lipid amide induced after-potentials were almost completely suppressed by 10μM TTX. These observations suggest that lipid amides modify axonal excitability by an exclusive action on sodium currents. Intracellular recording from unidentified axons in the desheathed nerve cord of Periplaneta americana were used to confirm and extend the above observations. Again, K^+ conductances were suppressed (to increase axonal input resistance and facilitate effective intracellular stimulation). (I) produced a sequence of dose and time-dependent effects in these cells starting with prolongation of spikes, depolarising after-potentials and repetitive firing and proceeding to complete conduction block. Spike-suppression developed slowly and was associated with pronounced axonal depolarisation. Action potentials with markedly modified kinetics could be restored by repolarising the axons with direct current.

For more detailed mechanistic studies mechanically dissociated neuronal somata from the thoracic ganglia of Schistocerca gregaria were used. Details of tissue preparation have been given elsewhere and when maintained in vitro for 8-10 hours such cells have been used to study GABA receptor pharmacology. However, very few (<10%) freshly isolated somata will produce all-or-none action potentials in response to current injection. Responses to depolarising (or "anode-break") stimuli most commonly observed are damped oscillations or varying amounts of outward rectification. By maintaining these axotomised neurones in vitro for longer than 24 hours their excitable characteristics can be markedly modified. Cells were incubated either in suspension, or after plating onto plastic petri-dishes, in 185mM NaCl, 5mM KCl, 2mM $CaCl_2$, 10mM Hepes, pH 7.0 supplemented with 100 ug/ml streptomycin and 100 U/ml penicillin. Neuronal survival and morphological preservation were increased further by the use of insect neuronal tissue culture media[3]. After 24 hours more than 85% of neurones impaled could be stimulated to produce fast, overshooting action potentials which were rapidly and completely blocked by 10^{-7}M TTX (Fig 1). Damage potentials and spontaneous firing where often encountered. 10^{-6}M deltamethrin evoked rapid and large depolarisations (accompanied by a reduction in cellular input resistance) in these modified spiking somata. These effects were blocked by TTX and were not observed in freshly prepared "silent" neurones. (I), applied at 5μM, evoked qualitatively similar changes in the excitable properties of these somata to those reported for the axonal preparations (Fig 1). After treatment with 0.5 mg/ml collagenase (10 minutes) and thorough washing whole cell patch recordings were obtained. Cells were voltage clamped using this technique and perfused with intracellular CsCl. (I) (5μM) slowed de-activation of Na^+ currents during a step depolarisation within 30 s of its application. After 10 minutes exposure large slowly-decaying tail currents were evident, peak evoked current was suppressed and negative shifts (5-15 mV) were apparent in the peak Na^+ conductance-voltage curve.

Conclusions:

A. Maintenance of axotomised insect somata _in vitro_ for 24 hours results in the development of Na^+-mediated electrogenesis. Na^+ currents can be modified in characteristic fashion by deltamethrin and TTX and can be studied using patch clamp techniques.

B. The lipid amides produce "pyrethroid-like" symptomology in multifibre preparations, single axons and voltage-clamped cell bodies which are consistent with their proposed actions on voltage dependent Na^+ channels.

1. Blade, R.J., Burt, P.E., Hart, R.J. and Moss, M.V.D. Pestic. Sci. 1985, 16 554.

2. Lees, G., Beadle, D.J., Neumann, R. and Benson, J.A. Brain Res. 1987, 401 267-278.

3. Beadle, D.J. and Lees, G. 1986 Neuropharmacol. and Pesticide Action - Ellis Horwood, 423-444.

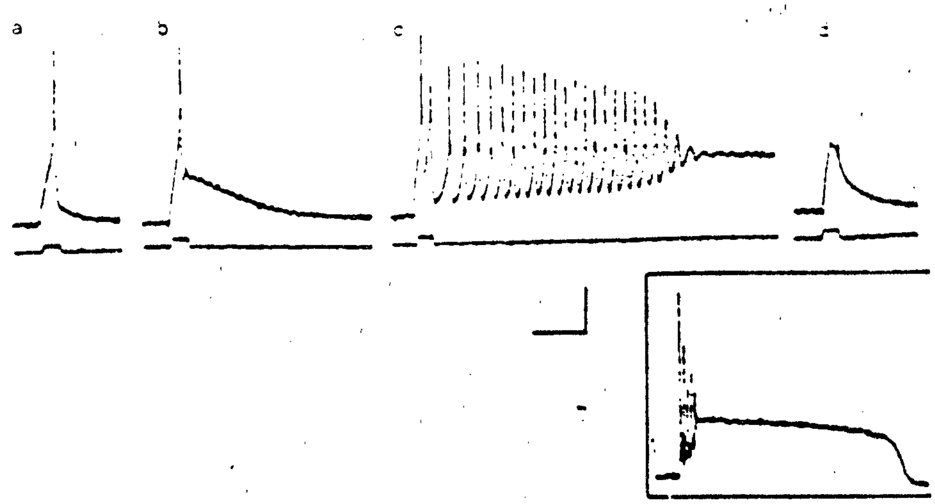


Figure 1. The actions of lipid amide (I) on a locust neuronal cell body which had been maintained in culture for 36 hours to induce spiking. Upper and lower traces represent transmembrane voltage and stimulus current respectively. Calibration: 20 mV, 50 ms (inset: 20 mV, 800 ms).

a. Control action potential profile.
b. One minute after application of (I) at 5μM a depolarising after potential is evident.
c. 2.5 minutes after application the evoked action potential is succeeded by a burst of spikes and a pronounced depolarising after potential exceeding 3 s in duration (Inset).
d. After 4 minutes exposure to the lipid amide, TTX at 0.1μM completely abolished these electrogenic responses. The toxin also blocks slowly developing depolarisations caused by prolonged exposure to high concentrations of (I).

TRIOXABICYCLOOCTANES: NEW ORDER OF POTENCY AND TWO TYPES OF ACTION

Christopher J. Palmer, Loretta M. Cole and John E. Casida

Pesticide Chemistry and Toxicology Laboratory, Department of Entomological Sciences, University of California, Berkeley, California, 94720, U.S.A.

1,4-Disubstituted-2,6,7-trioxabicyclo[2.2.2]octanes are a new class of insecticides[1,2] acting as $GABA_A$ receptor antagonists.[3] Further structural modifications have led to a new order of potency at both the organismal and receptor levels. Trioxabicyclooctanes and other cage convulsants that block the GABA-gated chloride channel appear to fall into two groups with different types of action. On comparing compounds of equal toxicity to mice, one group is at least 30-fold more potent than the other in inhibiting [^{35}S]t-butylbicyclophosphorothionate binding and GABA-stimulated chloride uptake in brain membrane preparations.

1. Palmer, C.J., Casida, J. E. J. Agric. Food Chem. 1985 33 976-980.

2. Palmer, C.J., Casida, J. E. Amer. Chem. Soc. Symp. Ser. 1987 356 71-82.

3. Casida, J. E., Palmer, C.J., Cole, L. M. Mol. Pharmacol. 1985 28 246-253.

EFFECTS OF SUBACUTE INTOXICATION WITH THIURAM ON BIOELECTRICAL ACTIVITY OF THE RABBIT'S BRAIN

Slawomir Rump, Marek Kowalczyk and Małgorzata Jakubiak

Department of Pharmacology, Military Institute of Hygiene and Epidemiology, 01-163 Warsaw, Poland.

Carbamates are well known for their broad biological activity, many of them are used as insecticides and fungicides. Their acute toxicity involves hyperstimulation of the autonomic nervous system and of central cholinergic functions. However, it has been shown that they inhibit also binding to GABA, opiate and muscarinic receptors in the brain , increase cerebral blood flow and decrease cerebral oxygen consumption , and depress Na,K-ATPase activity[3].

Present study was performed in order to elucidate the effects of popular fungicide of carbamate group, Thiuram (Bis-/dimethyl-thiocarbamyldisulfide/; TMTD) on bioelectrical activity of the brain after repeated administration.

Experiments were carried out on conscious male rabbits with chronically implanted electrodes into motor-sensory cortex (MSC) and dorsal hippocampus (Hip). Bioelectrical activity was registered on Grass Model 78 Polygraph and stored on Thermionic Racal 4FM Tape Recorder. Data evaluation was performed on IBM PC/AT machine by means of Fast Fourier Transform as was described elsewhere[4]. Thiuram dissolved in methylcelulose was given perorally during 15 days in a dose of 25 mg/kg (1/20 LD_{50}) daily.

It was found that administration of Thiuram resulted in MSC within 5 days in an increase of the whole power spectrum of activity (especially of fast activities). After 10 days a decrease of beta activity and an increase of slow activity was observed. After 15 days a further decrease of beta and alpha activities and an increase of delta activity was seen.

After 5 days in Hip only 5 c/s activity was increased. However, after 10 days power spectra of the whole analyzed record were increased (especially of fast activities, but also of 1 c/s activity). After 15 days power spectrum in Hip was similar to that seen in MSC.

These results suggest that Thiuram besides of cholinergic stimulation (increase of fast activity especially clear seen after 5 days of intoxication), resulted in signs of brain tissue damage (increase of slow activity, especially delta activity). Both effects were seen in MSC prior to MSC.

References

1. Speeg, K.V., Wang, S., Avant, G.R., Berman, M.L., Schenker, S. J. Neurochem. 1980, 34, 856-865.
2. Scremin, A.M.E., Scremin, O.U. Stroke 1979, 10, 142-143.
3. Stojanovic, T., Djuricic, B.M., Mrsulja, B.B. Experientia 1980, 36, 1348-1350.
4. Kowalczyk, M., Rump, S. Acta Physiol. Pol. 1986, 37, 241-251.

Mode of action of 1-phenylcarbamoyl-2-pyrazoline insecticides. Vincent L. Salgado, Rohm and Haas Company, 727 Norristown Road, Spring House, PA, 19477, USA

Though the outstanding insecticidal activity of 1-phenylcarbamoyl-2-pyrazolines has been known since at least 1975[1], there have been no data published on their mode of action. I will report data showing that these compounds paralyze insects by a voltage-dependent suppression of sodium current.

After injection of RH-421 (fig. 1) into the thorax of a cockroach (*Periplaneta americana*), there is a dose-dependent delay (6 hr at 0.5 µg, the LD_{50}; 20 min at 5.0 µg), followed by an initial period of hyperactivity and uncoordination, then prostration. While poisoned insects are prostrate, their legs kick as if trying to establish tarsal contact, and after a few seconds of kicking, all of the legs go into violent synchronous tremors lasting several seconds. This kick-tremor cycle repeats for an hour or so, after which time the prostrate roaches become very quiet, not moving for hours if left alone, but still able to perform a kick-tremor cycle if disturbed. The prostrate roaches persist in this state of volatile paralysis for four to six days, then gradually die. The symptoms produced by RH-421 are typical of all pyrazolines examined so far.

Although the convulsive tremors produced by RH-421 in cockroaches are striking, there is no evidence that the compound has an excitatory action at the cellular level, and, in fact, the only physiological effect I have found is a profound inhibition of activity in sensory nerves and in the central nervous system. Afferent activity in the crural nerve, the cerci and the abdominal stretch receptors was severely depressed or blocked in roaches prostrated by RH-421, as was activity in the meso-metathoricic connectives. Also, housefly larvae prostrated by topical treatment with RH-421 showed greatly depressed afferent activity in the segmental nerves. Further observations on roaches prostrated by RH-421 showed that restraining the legs in any way, or just providing tarsal contact, prevents the convulsive tremors. Taken together, these observations indicate that the paralysis caused by pyrazolines is due to block of activity in sensory nerves and in the central nervous system, while the convulsions may be a secondary effect, due to the overcompensation of some units for the decreased activity of others.

Block of sensory activity by RH-421, RH-529 (fig. 1) and a number of other pyrazolines was further assessed on several *in vitro* sensory preparations. When applied topically to a cockroach cercus, as little as 0.2 ng of RH-421 depressed cercal nerve activity noticeably, while 10 ng depressed the activity by 50% within 1 hour. *Manduca sexta* stretch receptors were blocked by 60 nM RH-421 within 30 minutes, while the cockroach and crayfish (*Procambarus clarki*) receptors required at least 2 µM for block.

Further studies of the mechanism of block were carried out on the slowly-adapting stretch receptor of the crayfish, because of its large size, using RH-529, which is more readily reversible than most pyrazolines. Following treatment of this cell with RH-529, the firing rate gradually decreased to zero, with no change in amplitude of the extracellularly-recorded axon spike, suggesting that initiation rather than conduction of the spike was being blocked. This was supported by careful intracellular measurements on crayfish giant axons that showed no effect of 50 µM RH-421 on the action potential. Current clamp experiments on the cell body of the crayfish slowly-adapting stretch receptor revealed that spike initiation in response to injected current was

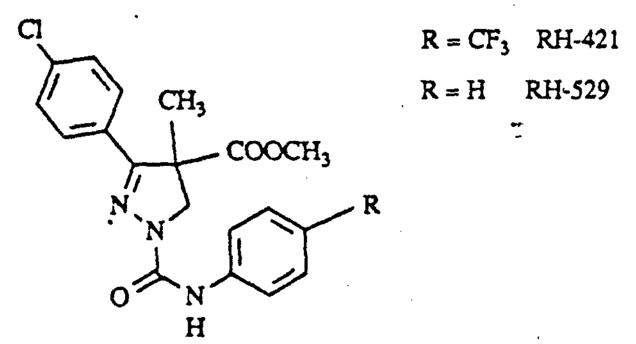
blocked by RH-529, with no change in resting potential or input resistance, suggesting that the sodium current was suppressed by RH-529.

Further experiments were done to determine why RH-529 suppressed the sodium current in the cell body but not in the axon. Because actively-firing stretch receptors have an average interspike membrane potential near -60 mV, compared to a resting potential of -90 mV in axons, I examined the influence of membrane potential on block by RH-529. An actively-firing stretch receptor was readily blocked by 10 µM RH-529, but when it was hyperpolarized to -80 mV by relief of stretch, it could, by injection of depolarizing current or application of tension, be made to fire normally for a few seconds, until it blocked again. Conversely, a crayfish giant axon in normal van Harreveld solution was not affected by 10 µM RH-529, but when its resting potential was reduced to - 74 mV by elevation of K^+_o to 125 mM, its action potential was reversibly blocked by 10 µM RH-529. These results indicate that the suppression of sodium current by pyrazolines is indeed voltage-dependent, and this voltage dependence can account for the apparent selective action on sensory nerves.

The voltage-dependent suppression of sodium current by pyrazolines was confirmed by voltage clamp studies on crayfish giant axons.

1. Mulder, R,, Wellinga, K., van Daalen, J.J. Naturwissenschaften 1975, 62 531-532

Fig. 1. Two pyrazolines used in this study.



INHIBITION OF LIVER MALATE DEHYDROGENASE BY METHYL PARATHION

S.P. Shukla and G. Tripathi
Department of Zoology, Banaras Hindu University, Varnasi-221005, India

Exposure of a sublethal concentration of methyl parathion (MEP), an organophosphate pesticide, reduced significantly the activity of cytoplasmic malate dehydrogenase (cMDH) and mitochondrial malate dehydrogenase (mMDH) of the liver of a freshwater catfish, Clarias batrachus. Marked ultrastructural changes were observed in the liver tissue of the MEP treated fish compared to the normal liver. Reduction in enzymatic activity in vivo could be correlated with the ultrastructural changes of the liver in situ. The in vitro experiments also demonstrated MEP-mediated reduction in the activity of cMDH and mMDH of the liver[1].

1. Tripathi, G. and Shukla, S.P. Biochem. Arch. 1988 (In press).

SPECIES DIFFERENCES IN THE *IN VITRO* INHIBITION OF BRAIN ACETYL CHOLINESTERASE BY MONOCROTOPHOS

Mohd. K.J. Siddiqui, F. Anjum, M.F. Rahman, S. Sultana, Mohd. M. Ali

Toxicology Unit, Regional Research Laboratory, Hyderabad-500 007
India

ABSTRACT

To develop safe and selective insecticides to avert an ecological and human health crises the determination of toxic kinetic property of a chemical is utmost important. The target enzyme pesticide interaction is of key significance in evaluating bioefficacy and species specific toxicity of a compound against target and non target organisms. The species differences in response to monocrotophos (phosphate of ciscrotonamide) of brain acetylcholinesterase (AChE) - a target of neurotoxicity was assessed by determining the *in vitro* inhibition in rat, mice, chicken and pigeon. Based on IC_{50} values, chicken brain AChE was found to be most sensitive followed by rat whereas mice and pigeon were almost equally sensitive to AChE inhibition by MCP. The relative resistance of these non target organisms may be attributed to the physiological adaptation to tolerate the toxic abuses of a polluted environment. The Lineweaver - Burk plot constructed to understand the mechanism of inhibition of rat brain AChE indicated that MCP decreased the apparent Vmax and increased the Km suggesting a mixed type of inhibition of enzyme. The results suggest that although there are many common features of Acetylcholinesterase in different species, there exist some differences in the response to monocrotophos and may be partly responsible for species differences in the toxicity of monocrotophos.

PESTICIDAL COMPOUNDS FROM *CEDRUS DEODARA*

Dwijendra Singh and Santosh K. Agrawal

Entomology Discipline, Central Institute of Medicinal and Aromatic Plants, Lucknow-226016, India

We have recently found that essentail oil obtained from Himalayan deodar (*Cedrus deodara* Roxb. Loud.) is a natural source of insecticidal principles against mosquitoes (*Anopheles stephensi* List.) under laboratory conditions[1]. This naturally occurring cheapest perfumery agent is also biologically active against pulse beetle[2] (*Callosobruchus chinensis* L.) and red cotton bug[3] (*Dysdercus koenigii* L.). Different chromatographic fractions of this oil evluated against pulse beetle and house flies (*Musca domestica* L.) have led to the purification of two sesquiterpenes possessing pesticidal activity viz. Himachalol (3%) and β-Himachalene (31%) based on essential oil weight[3]. These biologically active natural products of plant origin may possibly serve as a suitable prototypes for development of commercial insecticides.

1. Singh, D., Rao, S.M., Tripathi, A.K. Naturwissenschaften 1984 71 265-66
2. Singh, D. and Rao, S.M. Indian Perfumer 1985 29 201-4
3. Singh, D. and Rao, S.M. Current Science 1986 55 422-3
4. Singh, D. and Agrawal, S.K. J. Chem. Ecol.(USA) 1987 (In press)

DELAYED NEUROPATHY OF CYANOFENPHOS AND ITS METHYL ANALOGUE IN CHICKENS
S.A. Soliman, K.A. Osman, N.S. Ahmed, K.S. El-Gendy, and I.E. El-Shennawy*; Laboratory of Environmental Chemistry and Toxicology, Faculties of Agriculture and Medicine*, Alexandria University, Alexandria, Egypt
The delayed neurotoxic effect of the organophosphorus esters, cyanofenphos, O-ethyl-O-(4-cyanophenyl)phenyl phosphonothioate, and its methyl analogue, O-methyl-O-(4-cyanophenyl)phenylphosphonothioate, was studied in the chicken. Hens were orally treated by the compounds either once, or with subsequent daily doses. Some of the control and treated hens were sacrificed 24 hrs after the last treatment and their brain neuropathy target esterase (NTE) was measured. The remaining hens were used for clinical and histological examinations. Results demonstrated that methyl-cyanofenphos at a single dose of 200 mg/kg was able to produce delayed neuropathy in hens. This compound was also able to produce such effect in chickens given 25 mg/kg/day for 13 days. Cyanofenphos was also found to be neurotoxic to treated hens, but at somewhat higher doses. The effect was demonstrated by the standard clinical and histological changes and manifested by inhibition of NTE activity. However, the two compounds did not produce neurotoxic effect in chickens treated daily at 1 mg/kg for 180 days.

Molecular Biology and Pharmacology of Ionic Channels

M. Lazdunski
Centre de Biochimie
Centre National de la Recherche Scientifique
Parc Valrose, 06034 Nice Cedex
France

The channels which will be discussed include the voltage-sensitive Na^+ channel, the voltage-sensitive Ca^{2+} channel and K^+ channels including voltage-sensitive, ATP-sensitive and Ca^{2+}-sensitive K^+ channels.

(i) The voltage-sensitive Na^+ channel is the target of numerous toxins. The molecular biology of this channel will be briefly described together with the comparative pharmacological properties of Na^+ channels from mammalian and insect nervous system. The biochemical identification of a pyrethroid receptor will be discussed.

(ii) Ca^{2+} channel blockers associate with insect brain membranes. The unique pharmacological properties of receptors for Ca^{2+} channel blockers in insect will be described together with the identification of the protein subunit that is involved in the very high affinity of association for compounds in the phenylalkylamine series such as verapamil and desmethoxyverapamil (D888). Antibodies that react with another subunits of the Ca^{2+} channel in mammalian skeletal muscle, cardiac muscle and the nervous system also cross-react with a similar protein in the insect nervous system.

(iii) Tools which now permit to analyze the properties of voltage-sensitive K^+ channels (dendrotoxin and MCD peptide) of Ca^{2+}-sensitive K^+ channels (apamin and charybdotoxin) and of ATP-sensitive K^+ channels (sulfonylureas) will be described together with the structural properties of these channels when they are known.

DIFFERENTIAL EFFECTS OF FMRFAMIDE PEPTIDES IN HELIX TENTACLE MUSCLE

G.S. Bewick and G.A. Cottrell, Department of Biology and Preclinical Medicine, Bute Medical Building, St Andrews, Fife, U.K. KY16 9TS

The neuropeptide FMRFamide (Phe-Met-Arg-Phe-NH_2), first isolated from the clam *Macrocallista nimbosa* (Price and Greenberg, 1977), has since been shown to occur, along with several related peptides, in the circumoesophageal ganglia of the garden snail, *Helix aspersa* (Price, Cottrell, Doble, Greenberg, Jorenby, Lehman and Riehm, 1985; Payza, 1987). These neuropeptides have potent direct actions on the tentacle retractor muscle of *Helix*. The tetrapeptides (FMRFamide and FLRFamide) activate the muscle, inducing contraction after a marked delay on which phasic oscillations in tension are superimposed. This is in marked contrast to a smooth contracture evoked by exogenously applied ACh (Cottrell, Schot and Dockray, 1983). The three endogenous N-terminal extended peptides pQDPFLRFamide (pyroGlu-Asp-Pro-Phe-Leu-Arg-Phe-NH_2), NDPFLRFamide and GDPFLRFamide (N = Asn, G = Gly) all potently relax tentacle muscle (Lehman and Greenberg, 1987; Cottrell, Davies, Turner and Oates, 1988).

Since the C3 motoneurone, which innervates the tentacle retractor muscle, has been demonstrated to have "FMRFamide-like immunoreactivity", it was of interest to study the effects of the FMRFamide-related peptides on C3-evoked contractions. While FMRFamide itself was without any consistent effects, the N-terminally extended synthetic compound YGGFMRFamide (Tyr-Gly-Gly-) potentiated C3-evoked contractions (Fig. 1).

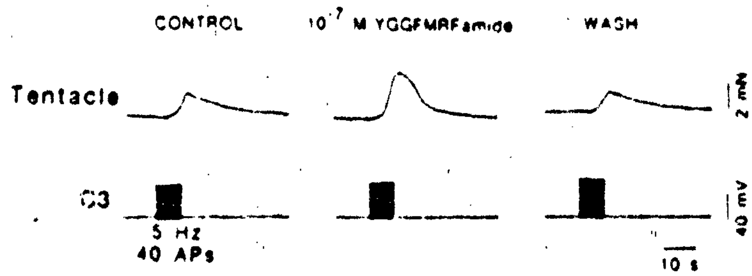


Fig 1

In contrast, the endogenous N-terminal extended peptides of the general structure X-DPFLRFamide (where X = N, pQ, G or S) all potently inhibited contractions elicited by C3 stimulation (Fig. 2). A scheme has been suggested as to how these peptides may interact in the behaviour of the animal (Lehman and Greenberg, 1987).

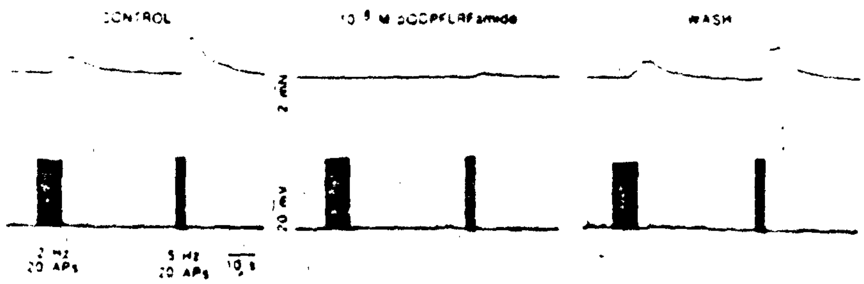


Fig 2

REFERENCES

Cottrell, G.A., Davies, N.W., Turner, J. and Oates, A. (1988) Actions and roles of the femerfamide peptides in Helix. In: Neurohormones in Invertebrates, Ed. Thorndyke, M.C. and Goldsworthy, G.J., Cambridge University Press, In Press.

Cottrell, G.A., Schot, L.P.C. and Dockray, G.J. (1983) Identification and probable role of a single neurone containing the neuropeptide Helix FMRFamide. Nature, 304, 638-640.

Lehman, H.K. and Greenberg, M.J. (1987) The actions of FMRFamide-like peptides on visceral and somatic muscle of the snail Helix aspersa. J. Exp. Biol. 131, 55-68.

Payza, K. (1987) FMRFamide receptors in Helix aspersa. Peptides 8, 1065-1074.

Price, D.A., Cottrell, G.A., Doble, K.E., Greenberg, M.J., Jorenby, W., Lehman, H.K. and Riehm, J.P. (1985) An extended FMRFamide related peptide in Helix: pQDPFLRFamide. Biol. Bull. Mar. Biol. Lab., Woods Hole 169, 256-266.

Price, D.A. and Greenberg, M.J. (1977) The structure of a molluscan cardioexcitatory neuropeptide. Science 197, 670-671.

Abstract for Neurotox '88 Meeting, Nottingham, April 10 15, 1900.

RECEPTOR-MEDIATED SIGNALLING USING SECOND MESSENGERS

Robert H Michell, Department of Biochemistry, University of Birmingham, U. K.

Cell surface receptors for hormones and neurotransmitters can be grouped into three fundamental classes: a) those that include within one multisubunit protein both a ligand recognition site and an ion channel that opens in response to agonist binding (e.g. nicotinic acetylcholine receptor, $GABA_A$ receptor); b) those that include within one protein both a ligand binding site and an enzyme catalytic site which is activated by agonist binding (e.g. the tyrosine protein kinases of the insulin and EGF receptors); and c) those receptors that transmit their activation, through guanine nucleotide binding G-proteins, to physically separate effector molecules within the plasma membrane.

The majority of the known members of this final group activate effectors which are enzymes (adenylate cyclase, cGMP phosphodiesterase, phosphoinositidase C (PIC), and maybe also other phospholipases C and phospholipase A_2) that change the intracellular concentrations of low molecular weight 'second messenger' molecules. Recent chemical and [illegible] cloning work has revealed a remarkable degree of homology amongst the members of this group of receptors. First, it appears likely that all G-protein-coupled receptors, from yeast to man and whatever the agonist and effector system employed, are members of a single evolutionarily related family of intrinsic membrane glycoproteins that span the plasma membrane seven times. Presumably there are unique features for each agonist-binding domain and for the domain that recognises each type of G-protein, but the receptors share some common mechanism for communicating the receipt of a stimulus through the protein between these two domains. Secondly, G-proteins are also a closely related group of (largely) heterotrimeric proteins, with the specificity for activation or inhibition of a particular effector system mainly dictated by the α-subunit. Stimuli that activate and inhibit adenylate cyclase transmit their effects, respectively, through G_s and G_i, and rhodopsin activation of cGMP involves transducin (G_t). The putative G-protein that regulates PIC has been named G_p, but is not yet identified. Indeed, receptor-stimulated activation of PIC is blocked by pretreatment with pertussis toxin on some cells, but not in others, so it seems quite likely that there is a small family of G_p's rather than a single G-protein that serves this function.

Having briefly discussed these general principles, I hope also to have time to talk briefly about recent advances in our knowledge of inositol lipids and phosphates in cell regulation.